Role of Ezrin in maintaining balance between cortical actin and stress fibers

A MS project report submitted in partial fulfillment
of the requirement for the award of the degree of

Master of Science

by

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1 Abstract

Ezrin is key to regulation of both membrane and cortical tension. By linking filamentous actin with membrane proteins, it helps forming various cellular structures like filopodia, growth cones, microvilli, lamellipodia, contractile furrow etc. These structures are crucial to cellular processes like migration, endocytosis and exocytosis, division etc. These developments happen through regulation of both membrane and cortical tension. Hence ezrin is crucial to tension regulation. It is known that ezrin organizes actin networks and forms above mentioned cellular growth structures. Also, it is reported that phosphorylating ezrin at specific site enhances rate of various cellular processes. Not only at cytoplasmic level, even in its inactive form ezrin can affect gene regulation and have a notable impact. Therefore, ezrin has a bigger role in all of these. Traction force is the force generated by actomyosin interaction and actin polymerization. This is transmitted to extracellular matrix, ECM, through stress fibers via focal adhesions which are local gathering of ECM proteins, membrane receptors and various cytoplasmic proteins. It varies from 1 to few nN depending on cell type. Cell cortex is known to be regulated not only in round spherical cells but also in well spread cells, by various physical properties like cell spread area, cell volume, and traction force too. It is reported that decreasing spread area increases cortical thickness and increasing traction force decreases cortical thickness. This happens via change of myosin II turnover rate, enhanced expression of cofilin and rate of actin polymerization. This elucidates the actin dynamics in MCA is regulated in response to change in spread area and traction force. We also observed in our lab that inhibiting ezrin increases traction forces. This implies strengthening of stress fiber. Thus, it compels us to ask further if ezrin plays an important role in regulation of actin dynamics and how. Hypothetically, is it achieved by redistribution of actin between stress fiber and cortical actin pool? To answer these questions, we inhibit ezrin by Ezrin inhibitor in CHO cells and to quantitate change in actin distribution we add Life-act mCherry fluorophore to fixed cells after inhibition of ezrin with keeping a control group added with same dye. Then observing the change in actin distribution in both stress fiber and cortex region we show that ezrin regulates stress fiber formation and cortical actin distribution through membrane tension modulation.

Keywords – Ezrin, Stress fiber, Traction force, Membrane tension.

2 Introduction

2.1 Actomyosin Cortex

Cellular processes like migration[1–3], division[2,4], tissue morphogenesis[5–7], cell differentiation and many more others involving cellular shape deformation are mainly governed by actomyosin cortex. This inherent property of the cortex arises precisely due to its structure. It is basically a pool of actin, myosin and many actin binding proteins[8,9] (ABPs) organized in a complex mesh. So, what property associated with cortex structure makes it govern key cellular processes?

Actin generally found in its filamentous form is an abundant protein in the human body. It is a very important cytoskeletal unit of cell and forms microfilaments which are around 7-9 nm[10] in diameter in single helix form (Figure 1.A). It performs very diverse but essential functions in every cell. Such as it present in a wide variety of cellular structures like microvilli, cell cortex, adherens belt, filopodia, lamellipodia, stress fibers (Figure 1.D) and contractile cell division furrow[11] etc. Also, it adheres cells by making tight bundles of filaments along with other proteins like cadherin. All of this function is possible because of the fact that actin filaments can bear tensile strength and also withstand it. Its monomeric form is known as G-actin, which has 4 protein domains consisting of α-helices and β-sheets and an ATP- binding cleft at the center[10]. This monomeric G-actin has two ends defined as follows. The C- terminal and N- terminal of the protein present on one side of the monomer define the end as positive end or barbed end. The straight opposite end to the positive side going through the center cleft is defined as the negative end or pointed end. Monomeric G-actins with help of nucleator proteins, like ARP 2/3, N-WASP, formins etc. pair up and create chains to produce filaments. Each pair of monomers use their positive end as a site of binding to the next pair by hydrolyzing their ATPs. As filament grows towards the positive end each strand winds around another forming helix and alongside stabilizing proteins such as tropomyosin, profilin stabilizes the filament. These steps help forming the filament and alongside different branching and bundling proteins like spectrin, filamin, ERM, play vital roles in strengthening filamentous actin. Also, disassembly can happen at both ends of the filament maintaining equilibrium of the process. All these processes all together play crucial roles in various cellular events. But keeping aside actin and its partner proteins who contribute to cortex regulation, non-muscle myosin II emerges as a key player in generating tensile forces and creating tension gradient.

Myosin II is a motor protein which essentially generates tension[12–14] by walking along actin filament. Its numerous presences in cortex actin mesh makes it an important part in cortical tension regulation. The answer to how it generates force and exhibits motor behavior lies in its structure. Myosin II consists of two domains (Figure 1.B). One long coiled coil domain, to whose N -terminal the other motor head domain is attached[12]. These two domains are made of 2 heavy chains and 4 light chains. Heavy chains consist of the head domain at the N-terminal and also the C-terminus coiled coil. The light chains are present at the neck region which is also called the lever arm. These light chains are the regulatory domains of myosin. The two light chains are known as regulatory light chain (RLC) and essential light chain (ELC). The motor head domain has ATPase site which is conserved throughout different families of myosin and another actin binding site. This actin binding site has a deep cleft which opens and closes in response to ATP binding. The ATP binds to the ATPase, gets hydrolyzed and then the product is released. In rhythm of the process, the cleft that binds to actin, opens and closes but doesn't cause large movement whereas lever arm goes through a swing of large angle of around 60 degrees. This swing then transmits through the c-terminal of the lever to helix coil which amplifies movement. Another striking property of myosin is that it is a bipolar filament forming protein. In fact, in muscle, it forms tight bundles of over hundred myosins in each with head motor domains hanging out from the bundles at opposite ends. And their long coiled coil tails make bundles in the center with strong interaction. And they are also arranged in particular fashion like staggered and parallel[13] method with respect to actin filaments which is a key structural aspect to generating contraction (Figure 1.C). Now once these numerous myosin motor heads attach to actin filaments, they swing their lever arms in response to ATP binding, hydrolysis and product release. And the actin filaments contract in response to that generating tension. Similarly in key cellular events myosin forms filaments and generates cortical tension in the same way.[15] . During cytokinesis myosin II becomes extremely organized making filaments in direction of the contractile ring whereas just before preset of the event it is sparsely distributed around the cell. Paramount importance of myosin makes it a key regulatory point in cortex.

So, cell cortex composing actin, myosin and numerous different proteins precisely controls mechanical cellular properties and coordinates many cellular events properly. Cell migration requires establishing different cell regions with contrast cortex properties. This is achieved by differing the density of actin, myosin and ABPs accordingly which results in regions with different cortical stiffness and generates the cortical tension gradient. The direction in which the cell moves is determined by the direction of the tension gradient.

Generally, when a cell migrates, it achieves so by crawling motion which requires a leading front and a trailing back. Studies show in moving cell, the leading edge shows softer cortical stiffness[2] while the trailing edge forms a stiffer cortex. Eventually this difference in cortical properties leads to cellular motion in desired direction.

Similarly in a dividing cell, cortex with contrast properties form distinct regions forming important structural preset to cell division. During contractile furrow formation which leads to making two cells from one, the distinctive regions formed by the cortical regulation are the polar region and the center region. The polar region consists of softer cell cortex while the contractile furrow consists of the stiffer cortex composition. This gradient then helps in division of cell [2]

Since actin cortex reflects a layer beneath plasma membrane, it was previously believed that this layer might hinder movement of vesicles toward or from plasma membrane. But people have shown that diminishing contents of actin cortex below a certain threshold led to inhibition of intake of granules. Then with advent of high resolution imaging techniques detailed dynamics of cell cortex become possible to translate. Change in thickness of cortex with respect to exocytosis and endocytosis proved the role of actin cortex in intake and secretion of vesicles[16] .

Other than dynamic movement of cell and processes involving force parameters, the actin pool of cortex also regulates special structures like microvilli, filopodia etc. The main aspect of actin pool which makes it able to procreate all those different structures is the shape and thickness of cortical actin pool. Normal cortex represents a relatively thin layer of actin all around the cell. Whereas lamellipodia contains thick cross linked dense actin pool. Microvilli contain long parallel actin filaments formed using actin linking proteins like α-actinin but filopodia formed by projections cross branched actin formed using ARP 2/3 complex. This indicates cortex changes actin dynamically by changing its constituents and orientations to perform various functions.

Therefore, cortex regulation is one of the fundamental control points in cellular morphogenesis and mechanical events. But in all of these, maintaining cell shape and integrity in cellular membrane is also very crucial for survival of cell. Thus, the cell membrane and cortex go hand in hand in all these regulatory processes. This becomes possible by the critical adhesion of cell plasma membrane to the cell cortex. There are different and numerous key proteins which help in maintaining the membrane cortex attachment. ERM family of proteins are one of those linking proteins[9]. Among the three, Ezrin is one key protein which not only

helps keeping MCA strong and stable but also key to other important biochemical pathways like signal pathways and regulatory pathways[9,17].

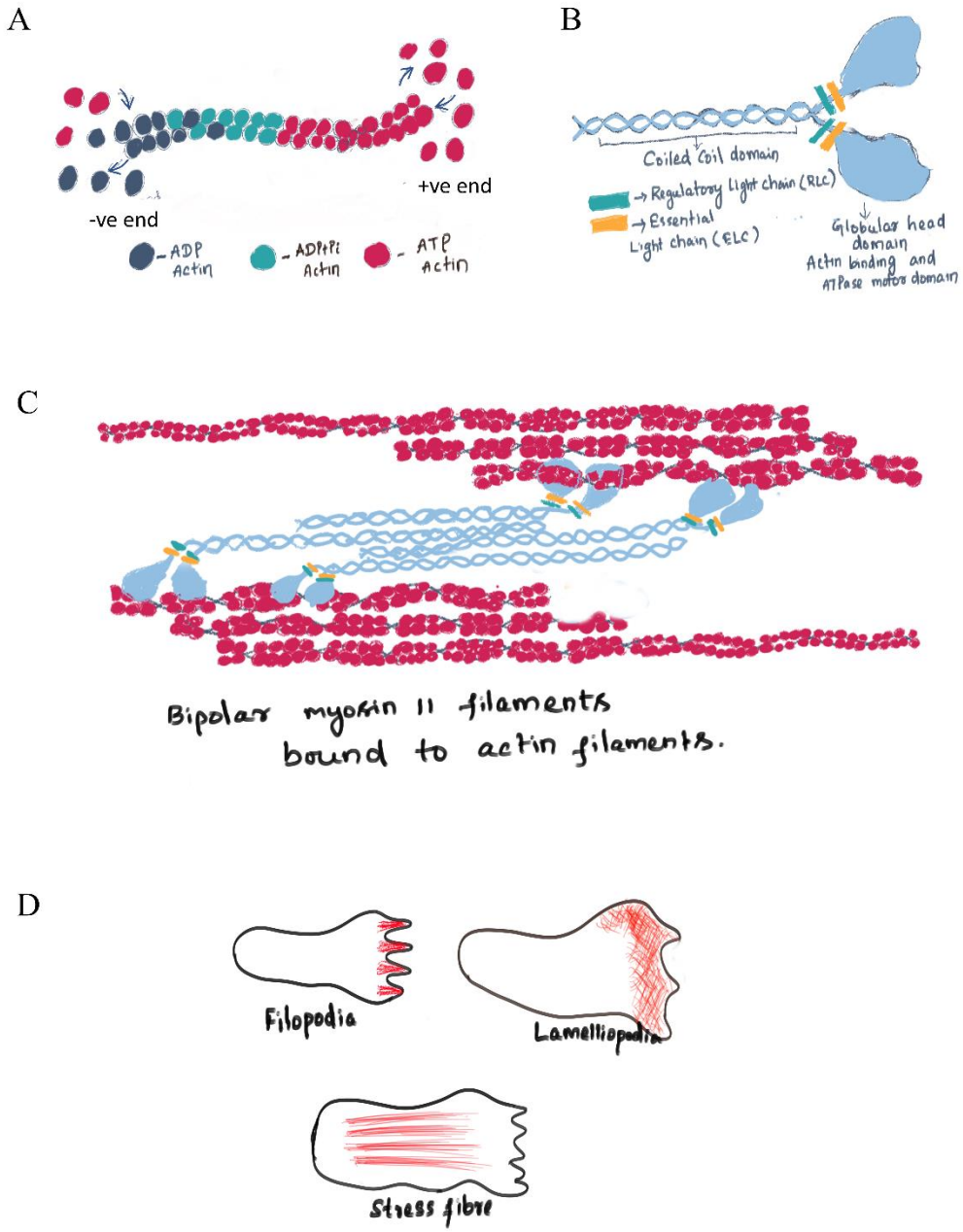

Figure 1 A. Formation of actin filament by polymerization and dissociation. B. Structure of Myosin II. C. Actin filaments crosslinked by myosin II bundles. D. Different cellular structures formed by actin filaments.

2.2 Ezrin and Membrane Cortex Attachment

Ezrin protein with molecular weight around 70-85 kDa and consisting around 587 residues contains 3 domains made up of several α-helices and β-sheets (Figure 2.A). Starting from the N-terminal, residues 1- 296 forms the FERM domain which contains 3 subdomains called F1, F2, F3[18,19]. This domain is also known as N-terminal ERM association domain(N-ERMAD) as it is the site which generally binds to membrane integral proteins. Residues 297-469 forms the long coiled coil domain which consists of two antiparallel long helices and is called central helical domain[18]. It consists of 3 subdomain, α-1H, α-2H and α-3H. And the end domain is formed by residues 470-587, and since it consists of the C-terminal site, it is called the C-terminal domain or CTD. This domain is responsible for binding to actin filaments[18].

The FERM domain consisting of 3 subdomains has many clefts with specific biochemical properties. This domain is an evolutionary conserved domain of around 40 kDa[19] molecular weight with a tyrosine amino acid at residue 145 potentially available for phosphorylation. These 3 subdomains are organized in a triangular shape[20,21] making interaction with each other , and these interactions are the binding force of the stable FERM domain. Interactions include hydrophobic interactions, salt bridges and charge force interaction[19]. It has been reported that F3 domain interacts with F1 using a positive charged face conserved within the cleft present between them while it interacts with F2 with a negatively charged face present in the cleft between them. On top of these clefts, these subdomains are site to various important conserved residues which helps the FERM domain not only attach to cell membrane integral proteins but also perform various signaling functions and some other functions which are yet not known. It also binds to the CTD domain forming an inhibited or inactive state of ezrin protein. This state is crucial in regulation of MCA and tension regulation in both cortex and membrane. Along with that the class of proteins those bind to FERM domain consist numerous types such as CD43, ICAM, matrix metalloproteinase (MT1-MMT). These different class of proteins according to their structural variation bind to different subdomains of FERM in grooves by using specific interactive method corresponding to those grooves. Such as one group of proteins extend its β-sheet and bind to β5F3 sheet present in F3 domain groove above α1F3. Similarly other class of protein binds to β2F1 sheet present in F1 subdomain by extending their β-sheets. This interactive FERM domain makes it a hub to signaling pathways like Hippo signaling pathway, lats kinase pathway and various other still to be discovered[18]. One of the striking properties of ezrin is resemblance between CTD binding to FERM domain and other proteins binding to FERM domain making it an

important regulatory domain since all of the proteins would be blocked in this closed state conformation. Hence FERM domain is an important and functional domain of ezrin.

The central helical domain consisting 3 α-domains, α-1H, α-2H and α-3H, with a hinge present between the later α- helices is bound to globular FERM:CTD complex and is conserved through evolution with selective length implying functional importance. The long antiparallel colic coil structure is maintained by the heptad repeat which only breaks at the hinge but again continues in the α-3H domain. Heptad sequence is a mixture sequence of 7 amino acids which consists of particular types of amino acids at specific positions[22]. It is generally represented as

$$\begin{matrix} a\ b\ c\ d\ e\ f\ g \\ \text{H P P H C P C} \end{matrix}$$

Where H represents hydrophobic amino acid, P represents polar amino acid and C represents charged residues. The coiled coil structure constitutes 7 heptad repeats from C-terminal end of α-2H, and complete α-3H. Also, it has been seen, though the N-terminal of α-2H partially positioned inside FERM domain which is a condense region, still it contains 2 heptad repeats in its structure. But within the hinge, the hydrophobic residue repeats at position 'a' and 'd' is not maintained and the heptad sequence breaks down and this makes it unable to form coiled coil. Altogether they form a long coiled coil structure presumably helpful in formation of swapped dimer of ezrin which may have certain importance in its functionality.

The CTD domain is observed to vary across families of ERM proteins. Its total sequence can be divided into two categories: proline rich residues[18], whose function is still not understood properly and a β-sheet which is present near the C-terminal. This domain is mainly responsible for two important functions. One is to bind with actin filaments and actin binding proteins, and another is to masking/unmasking the FERM domain. In the second scenario, it does so by forming a reported two dimensional flat structure covering the FERM domain as an outer coat. The proline-rich segment of the CTD forms a hairpin-like structure that goes into a groove between F1 and F3 domain of N-ERMAD and the β-sheet extends to β-sheet of the F3 FERM subdomain. These two structures when present bound to FERM hinders different sites for binding like PIP_2, and some α-helices domains with potential binding tendencies[18].

The FERM:CTD complex is an important part of ezrin protein because its close/open state formation becomes key to regulation of other protein binding and hence many signaling pathways. The stability of FERM:CTD interaction is possible because of interaction between several hydrophobic faces of α-helices and hydrophobic pockets present inside grooves of FERM subdomains, especially F2 and F3. Also, the interaction involves some electrostatic interactions. In addition, it has been seen that the CTD domain contains some intra-chain

structures which only form after binding to the FERM domain. Strikingly it is observed that all these interactions are conserved through evolution suggesting key functional aspects of CTD domain and ezrin.

Moreover, the C-terminal residues of CTD are involved in specific interactions with actin filaments and ABPs. Studies have shown that ezrin binds to actin but not tubulin signifying its role in cortex. Ezrin is also found to be connected with the barbed end of actin and actin nucleation protein ARP 3. Additionally, Studies show the threonine residue at position 567 is key to activation of Rho signaling pathway and that sequentially promotes cortex actin cytoskeleton formation[23] Therefore, ezrin is a crucial structural and functional protein for maintaining structural integrity of MCA as well as for key regulation of mechanical parameters of membrane and cortex.

Ezrin, as described above, is involved in a wide range of functions starting from signaling pathway regulation to controlling various physiological processes in a group of cells. Since activation happens by phosphorylation at various sites of ezrin, potential molecular inhibitor targeting to mask that part can be fundamental in many pathways. Generally different kinases like protein kinase c, MRCK, AKT activates ezrin by phosphorylating PIP_2 , which generally binds to ezrin on its FERM domain.[20] Thus, ezrin inhibitor molecules, like NSC668394, named 7-(3,5-dibromo-4-hydroxyphenethylamino)quinoline-5,8-dione binds to same site[24] resulting a mask. And as phosphorylation is essential for activation of ezrin after which CTD does binding with actin, FERM binds with membrane proteins etc., inhibition by masking stops these processes too. FERM domain is also crucial in signaling pathways like Rho. Therefore, inhibition of FERM downregulates these pathways.

It was previously believed that ezrin is a downstream molecule in Rho signaling cascade but recent studies showed ezrin to be rather involved in upstream regulation of some proteins, which regulate Rho activity[9,25,26]. Since Rho signaling finally affects and control cortical cytoskeletal distribution, ezrin inevitably becomes key regulator of cortical actin pool. Many membranes signaling proteins like Patched(ptc.)[9] not only get linked to receptors by ezrin protein but also get the assemblies of receptor complexes which are formed with the help of regulatory processes involving FERM domain, like interaction with PDZ-domain which contains adaptor protein EBP50 and E3KARP[18]. Ezrin and other ERM proteins are also involved in maintaining polarity in developing cells. Depletion in concentration of ezrin protein shows mislocalization of important development factors like staufen and oskar mRNA leading to failure in polarity establishment in oocyte[27,28]. Since there is no direct involvement of ERM proteins in deciding the localization of these polarity establishing proteins, it is proposed that

they help in organizing cytoskeleton in polar faces which then helps in localization and establishment of polarity. Similarly in mitotic division, ezrin helps in regulation cortical actin density and that leads to generation of tension gradient and promotes cell rounding. Experiments involving depletion of ERM proteins showed abnormalities in mitotic process. Other key processes involving ezrin are lumen morphogenesis, epithelial morphogenesis, metastasis etc[9]. Even in its dormant form, not only it masks potential FERM domain interaction but also regulates stress gene effect. Study shows that inhibiting ezrin results in upregulation of various stress genes like PTGS2, DDIT3 etc[29]. The diverse and key functions of ezrin are remarkable.

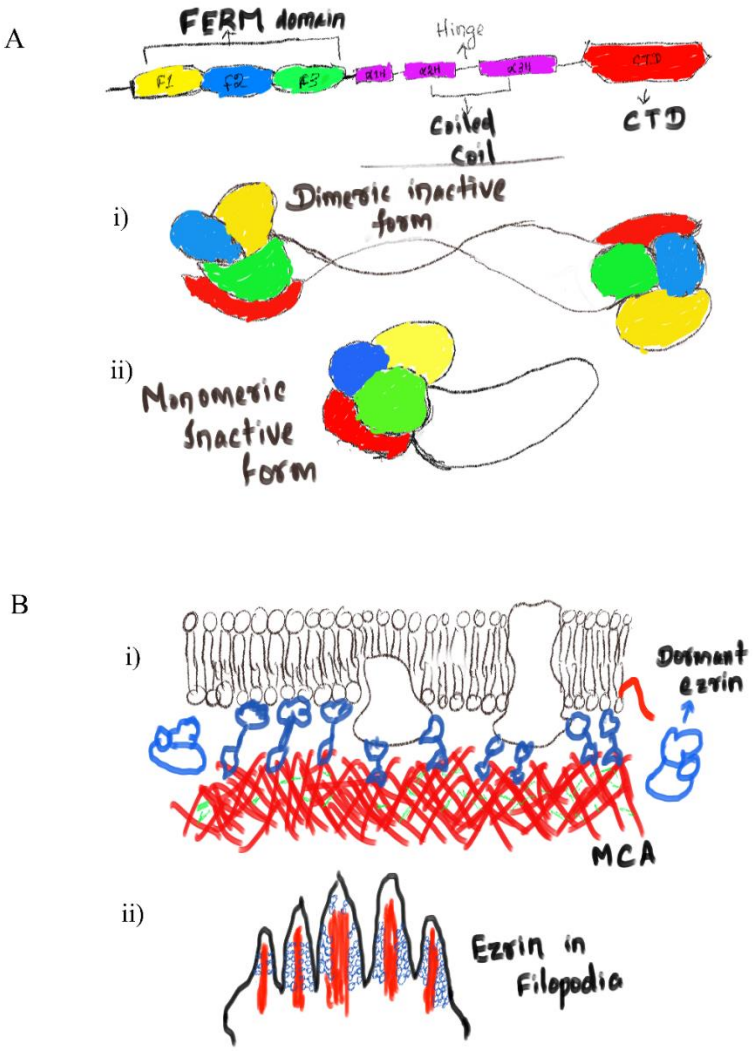

Figure 2 A. Ezrin protein and its 3 subdomains. Inactive form in two possible conformations i) Two ezrin molecules masking each other in dimer confirmation (inspired from imref0) ii) Ezrin masking itself in monomer confirmation B. i) Ezrin linking membrane to cortex. ii) Ezrin helping formation of filopodia.

2.3 Membrane Tension, Cortical Tension, and Traction Force

Plasma membrane is a lipid bilayer with many transmembrane and membrane integral proteins, as well as filled with many cholesterol rafts and lipid molecules of other kinds. It behaves like a fluid mosaic[30] in which lipid molecules are seamlessly dancing due to thermal fluctuations and are diffusing randomly by interchanging positions[31] among them. Since the plasma membrane is the boundary of cell, they are constantly exposed to various mechanical forces. Starting from osmotic pressure generated because of the difference in external and internal environment of the cell, to experiencing forces from other cells in cell-cell adhesion[32]. All these external forces cause the membrane to generate tension. Other important components raising membrane tension are interaction with the acto-myosin cortex present beneath it, friction present in the substrate on which cell migrates, forces arising due to adhesion to other cells, friction generated by interaction with various extracellular proteins etc. Therefore, membrane tension is a crucial parameter representing mechanical properties of a cell and thus it is regulated for maintaining cell shape, integrity and for control of a lot of cellular processes involving physical deformations of membrane.

General definition of tension is the force experienced per unit area. Similarly, to apply the concept of tension to plasma membrane we have to imagine a infinitesimally small patch of membrane with imaginary boundaries and to make it simple we consider a symmetric square patch. So now the notion of membrane tension is translated as the force acting tangential to the patch per unit length of the boundary. Hence membrane tension is expressed as N/m. But to experimentally determine the value of membrane tension is a challenging task. Wolfgang Helfrich in 1973 developed a method to account for various properties of cell membrane including membrane tension. Lipid bilayer can also be considered as an elastic entity and hence should possess elastic energy. Helfrich described the elastic energy of membrane using membrane tension (σ), bending rigidity of membrane(κ), change in area (ΔA) and local curvature (C). The bending rigidity of membrane can be described as the force opposing change in curvature of membrane[33]. The equation describing the relation is

$$\boldsymbol{E} = \boldsymbol{\sigma \Delta A} + \oint \frac{\kappa}{2} C^2 dA \qquad \textbf{(1)}$$

This equation can be applied for tether pulling experiments and we can finally simplify that σ is $F_0/ 4\pi R$ [34] where F_0 is the force with which tether is pulled and R is the radius of curvature.. Moreover, fluctuation model which uses power spectrum density[35–38] can also be used to calculate the membrane tension using fluctuation generated at membrane.

Membrane tension regulates plethora of cellular functions like cell spreading involving addition of lipid bilayer, vesicle trafficking which involves addition and withdrawal of lipid bilayer, establishing cell polarity, membrane fusion etc. Some studies report membrane tension can propagate globally and regulate at a distance. One model shows, during cell motility, membrane tension at one location generated due to active polymerization of actin leads to inhibition of formation of protrusion at distance site[39]. But some other studies show membrane tension produced locally take longer time to propagate distant location. These seemingly opposite views open up interesting directions to membrane dynamics understanding. One possible explanation is, membrane tension propagation is impeded due to various membrane proteins and actin cytoskeleton present beneath[32]. These constituents vary cell to cell. Therefore, membrane tension regulation is a complex process and needs more insight.

Traction is defined as the force per unit length exerted in the tangential direction of substrate by the cell. The origin of cell traction force is the tensile force generated in actin filaments by myosin II motors. Then this force is transmitted to ECM through focal adhesions (FA). FA is formed by interaction of transmembrane proteins with extracellular ligands[40]. These transmembrane proteins and integrins on the interior of cell are linked to multiprotein assemblies and the cytoskeleton. These multiprotein assemblies contain various ABPs and cytosolic proteins like FAK, vinculin, actinin, myosin, paxillin, talin, zyxin, tensin etc. FAs anchor the cell to substratum and are involved in mechanical & biochemical signaling[41].

2.4 Motivation and objectives

Cortex and membrane are two sides of a coin. They are involved in important cellular mechanics and regulation of their tension profile becomes key to these processes. During cell migration the cortex regulates its tension profile by dynamically changing its actin distribution and creates gradient. Simultaneously membrane tension propagates globally regulating protrusions as well as maintaining shape. During cell division cortical tension gradient makes the contractile ring while membrane tension protects the cell shape. Therefore, actomyosin cortex and cell plasma membrane go hand in hand in the regulation of various cellular

processes. Since ezrin is the important protein present in MCA, it also holds key regulatory possibilities. Studies have already shown that ezrin regulates membrane tension[42] and affect cortex by actin polymerization in structures like growth cones [43]. But it is not clear how ezrin affects the cortex globally. Though local growth structures like growth cone involves role of ezrin to some extent, it also uses tension gradient generated by cortex. Therefore, regulation of cortex still needs more insight for better understanding.

A study from our lab shows physical parameters can regulate cortical actin distribution[44]. Specifically, cell spread area and traction force regulates cortex thickness. By micropatterning cells with different patterned mask, high spread area was achieved with close replica of in vivo substratum and also cells with different spread area were cultured. Quantitating cortical thickness in those cells revealed an inversely proportional relation between them. Decreasing spread are showed increased cortical thickness and it does so by changing myosin II turnover rate and enhanced cofilin expression. Similarly increasing traction force by varying substrate rigidity while keeping spread area constant resulted in decrease in cortex thickness. This shows cortex regulation involves physical parameters like cell spread area and traction force. **Since traction is brought about by stress fibers, we wanted to understand if stress fibers may affect cortex at higher-up planes through the membrane and MCAs.**

Interestingly, recent results in the lab showed that inhibiting ezrin increases traction stress. Since traction stress regulates cortical actin distribution, it compelled us to further investigate whether ezrin regulates cortical actin distribution. We know traction force is transmitted through stress fibers to focal adhesion. Therefore, we investigated if stress fiber is also involved in actin redistribution through mediation of ezrin. **Hence, we aimed to understand how ezrin regulates actin distribution at the cortical layer.**

Therefore, the specific objectives of this study were:

1. **Imaging acto-myosin cortex at the basal cell membrane using Total Internal Reflection Fluorescence Microscopy**
2. **Setting up quantitative analysis of actin intensity and area of stress fibers.**
3. **Setting up comparative analysis of stress fiber and stress-fiber-free cortical regions**

4. **Using ezrin inhibition by NSC668394 (a quinoline based molecule) drug to understand the role of phosphorylation of ezrin at threonine, T567 in distribution of actin**
5. **Using siRNA to reduce ezrin expression to understand the role of total ezrin in determining actin distribution**

The quantification of intensity would lead us to establishing distribution properties in different conditions. But to imply more about the rate of distribution we aimed to find ratios of change in intensity of stress fiber region to cortical actin region. To further support our investigation, we looked upon total f-actin distribution in cells, total stress fiber intensity distribution and spread area distribution.

3 Materials and Methods

The aim of this work was to quantify any subtle change in the stress-fiber vs. cortical actin distribution that might be brought about by ezrin. Towards this, the experiment planning required

1) Control on cell spreading

   This is needed because change cell shape can affect distribution of stress fibers. This was brought about by micropatterning in which the pattern chosen fixed the cell width while the cell is still free to choose its length.

2) Quantitative imaging of actin close to the basal membrane

   This is needed because only if a thin section of the basal cortex is sampled can the stress-fiber-free region be demarcated from regions with stress fibers. Thick optical sectioning will have over-representation of amount of cortical actin per pixel since stress fibers are relatively this ~ 100 nm. We use TIRF microscopy to circumvent this problem. The penetration depth of TIRF was kept at ~ 70 nm.

The following sections present in detail the concept and protocol of the different methods learnt and used.

3.1 Cells

Chinese Hamster Ovary (CHO) cell is an epithelial cell line derived from ovary of Chinese hamster. Its first subclone was derived by T.T. Puck in 1957. They are widely used in medical and biology research. CHO cells are now considered as gold standard in model systems because they enable accurate production of proteins with proper folding and translational modifications. They are easy to culture on a large scale and suitable to grow under required conditions. Because of their epithelial origin they are most suitable for study of cortical and MCA proteins.

3.2 Micropatterning

Cells are sensitive to the physical parameters like geometry, rigidity and other mechanical parameters in their microenvironment. Therefore, to control these parameters recent developments in micro engineering tools have been proved useful. So, to suit our needs

these tools help us restrict spread location, and composition of the substrate in particular areas where cells attach and are called micropatterns. Especially in our experiment we needed well spread cells to extensively use their cytoskeletal machineries. Therefore, we used Line 10 pattern which consists of parallel tracks of 10 μm breadth and indefinite length so that cells can spread horizontally but are restricted in width. Materials needed for carrying out the procedure are

- Poly-L-lysine grafted polyethyleneglycol (PLL-g-PEG)
- Fibronectin
- Glass Coverslips
- Ethanol (95%), Isopropanol, acetone, water
- UVO Cleaner
- Photomask with Line 10 pattern

X-ray photoelectron spectroscopy of carbon 1s of PLL-g-PEG before and after deep UV screening using a coverslip explains how it adsorbs protein after UV irradiation. A 'C-O-C' component of PEG whose C 1s spectrum shows high peak at 286.8 eV, disappears after deep UV irradiation. This is the component which causes protein repelling and hence doesn't absorb protein if not irradiated with UV. And after UV irradiation PLL-g-PEG shows a characteristic peak corresponding to carboxyl group which attracts protein. That in turn confirms strong protein adsorption onto glass coverslip. Then fibronectin gets adsorbed to patterned areas as those are the areas irradiated with UV. Fibronectin mimics ECM texture which helps cells attach by forming focal adhesion. Hence cells attach to restricted areas only (Figure 3).

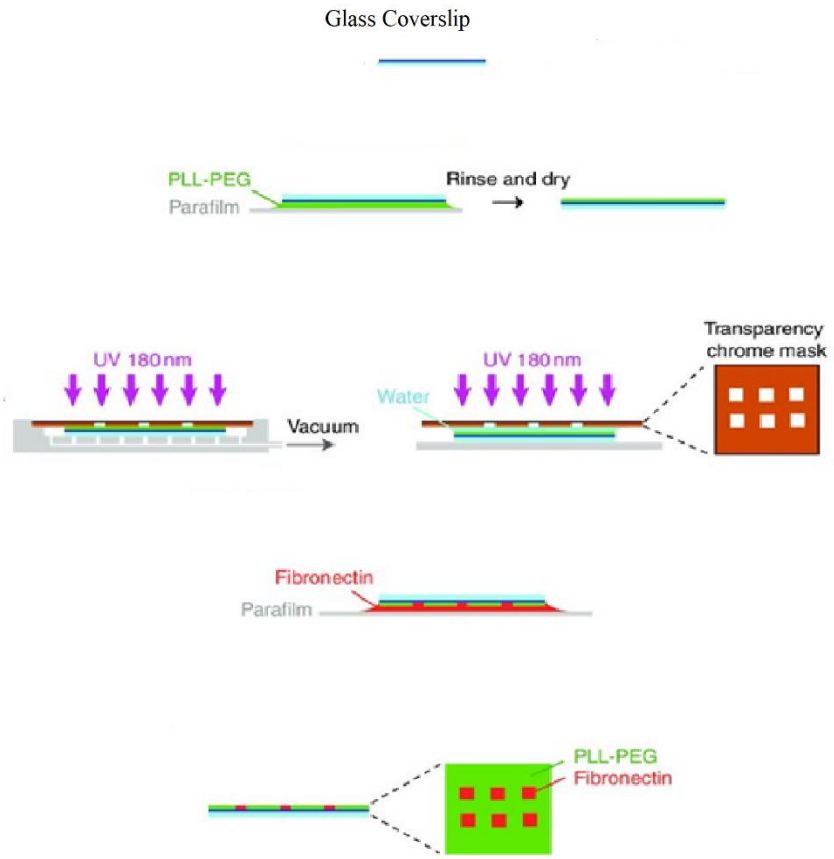

Figure 3 Schematics of micropatterning [45]

The standard protocol followed is

1. Coverslips are first etched in solution of acetic acid and EtOH in ratio of 1:19. Then they are transferred to 95% EtOH solution.
2. Then etched coverslips are air dried and UV treated in UVO cleaner box for 5 mins.
3. 20 µl of 0.2 mg/ml PLL-g-PEG is put on a piece of parafilm and coverslips are put on those drops of PLL-g-PEG with UV shined side towards the droplet. Those are incubated for 1hr.
4. Photomask is washed with Isopropanol, acetone and water respectively and then dried using a dryer. Then it is UV treated with chrome side exposed towards UV for 5 mins.

5. PLL-g-PEG coated side of coverslips are then stuck to pattern or chrome side of photomask using 2 µl of water and exposed to UV for 5 mins by placing them upside down (the silver mirror part facing upward) in UVO cleaner.
6. Coverslips are then carefully removed by floating them off with water.
7. 20 µl of 25 µl/ml Fibronectin is put on a piece of parafilm and coverslips with side attached to the pattern is placed on droplet making sure no air bubble remains. And incubated for 1hr.
8. Now these micropatterned coverslips are used in making cell culture dishes.

3.3 Total Internal Reflection Fluorescence Microscopy (TIRFM)

TIRFM is a powerful technique which allows selective imaging and is efficient when desired structures are close to cell surface. The basic principle guiding TIRFM is the principle of total internal reflection which basically translates to incident light completely reflecting back without refracting. That gives rise to evanescent field waves near the interface in the medium from which light gets reflected.

When light goes from one medium to another it follows Snell's law to determine the angle of reflection and refraction. Let's assume light ray goes from a medium with refractive index of n_1 to a medium with refractive index n_2, the equation that governs its angle of reflection and refraction is

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \qquad (2)$$

Where θ_1 and θ_2 is the angle of incidence and refraction respectively. These angles are defined with respect to the normal to the boundary of two mediums. The angle of refraction can be written as

$$\theta_2 = \sin^{-1}\left(\frac{n_1}{n_2} \sin(\theta_1)\right) \qquad (3)$$

So, for total internal reflection the angle of refraction must be at least $\pi/2$. Putting this constraint in the equation we get

$$\sin \theta_1 \geq \frac{n_2}{n_1}$$

$$\Rightarrow \theta_1 \geq \sin^{-1}\left(\frac{n_2}{n_1}\right) \qquad (4)$$

The angle when the equality holds is defined as the critical angle of reflection. At an angle equal to this value or above results in total internal reflection. But simultaneously an electromagnetic evanescent field is formed at the interface and that propagates parallel to the

interface. Rays closest to the interface in the direction of normal of the refracting medium but decays exponentially (Figure 4.B) which is governed by the equation:

$$Re(E_T) = E_{0T}cos((k_T sin\theta_T)x - \omega t)e^{-Az} \quad (5)$$

Here T subscript describes "of transmission", E is the electric field, k is a constant, ω is the frequency of light, t is time, A is an imaginary constant and z is the direction of propagation which is the normal in refractive medium. This is the electromagnetic wave that is propagated into the refractive medium is known as evanescent waves. The physical explanation for this phenomenon is that electric field is continuous at the interface of mediums. The length of this decay can be much shorter than the wavelength of light. Therefore, it causes energy propagation in some nanometer length scale. TIRFM uses this evanescent wave to excite fluorophore present nearest to the interface. The evanescent field energy is absorbed by the fluorophore and it momentarily goes to a higher electronic energy state. Within (usually) nanoseconds it comes back to the ground state lower vibrational state and then comes back to ground state illuminating fluorescence. Since only the molecules nearest to the interface are illuminated, a great signal to noise ratio is achieved and it helps in analyzing objects of interest properly.

TIRFM setup includes a laser, a dichroic mirror, an objective and a camera detector. Generally the objective is added with immersion oil to make the propagation of laser beam better onto coverslip surface. The important precaution in setting up the TIRF setup is the proper selection of objective lens. The reason behind this choice can be explained using numerical aperture of the objective. NA of an objective is

$$NA = n_1 sin(\alpha_{max}) \quad (6)$$

Where α_{max} is the maximum angle achieved by the incident ray. The depth of evanescent field can be described by an equation as

$$d_{min} = \frac{\lambda}{4\pi}\sqrt{(NA^2 - n_2^2)} \quad (7)$$

Here λ is the wavelength of light and n_2 is the refractive index of 2nd medium. So, if NA is smaller than n_2 then evanescent field depth would be imaginary. So TIRFM won't happen. Therefore, NA of objective should be higher than that of refractive medium. Our setup uses an objective of NA 1.49 and that of PBS, the medium used for cell imaging is around 1.33. The evanescent field depth or also known as penetration depth can be fixed in the Olympus IX81 microscope we use and is kept at 70 nm. And also, we keep the off-set angle to be at -

4.5°. In this setting we observe stress fibers are clearly demarcated with a great signal to noise ratio and hence keep these settings as standard for all our imaging.

A DPSS 488 nm laser is used as light source and a CMOS camera of 1 pixel mapping to 65 nm is used with exposure time maintained at 500 ms for capturing of each image (Figure 4.C).

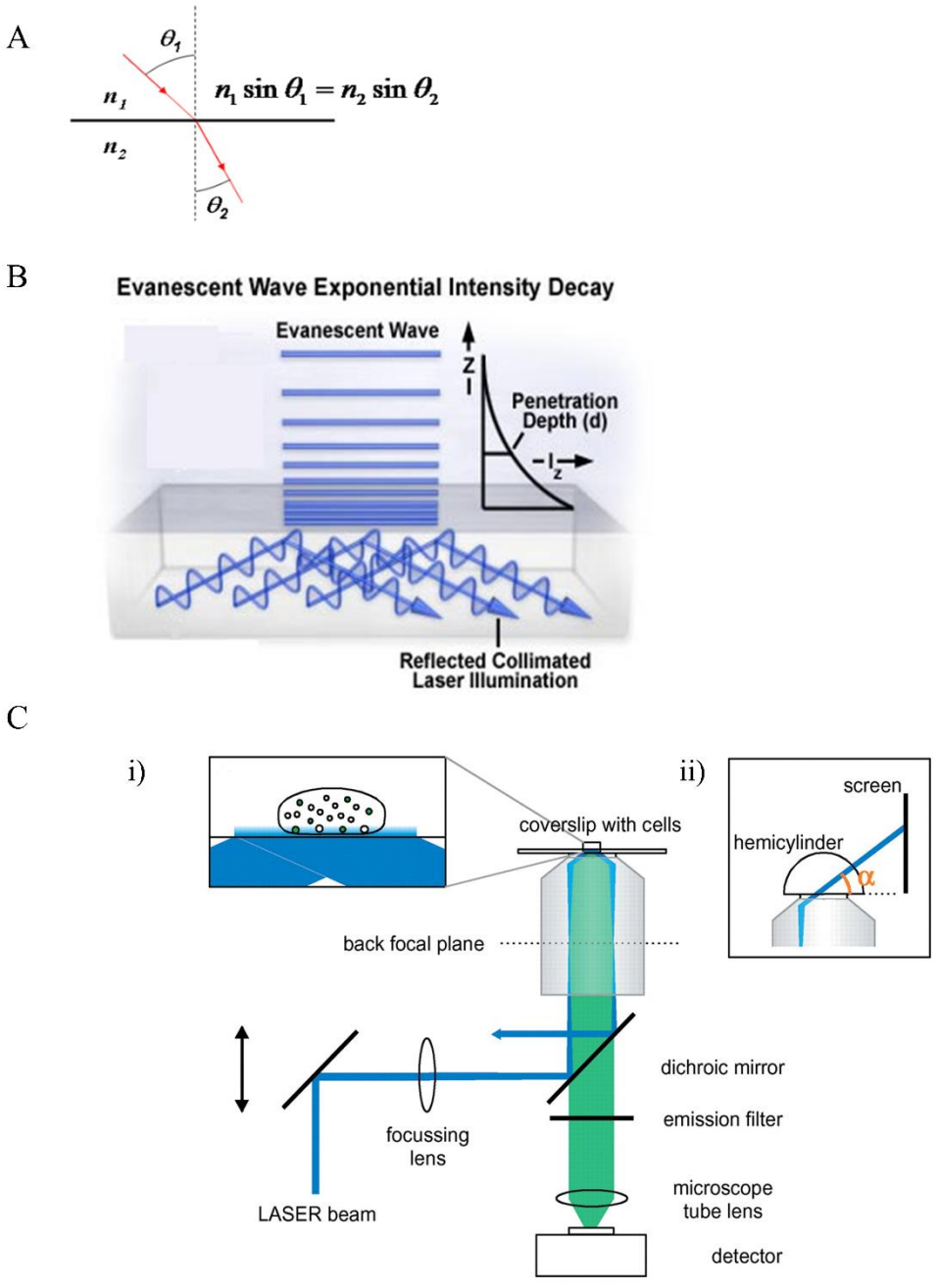

Figure 4 A. Snell's law describing refraction. (imref₁) B. Evanescent wave formation and its exponential decay.(imref₂) C. Ray diagram of TIRF microscopy describing i) incident and reflected ray and ii) α_{max} – maximum angle implying numerical aperture [46]

3.4 Cell culture, Fixation and Staining

Life systems require proper nutrient supplements to grow and at the same time periodic cleaning of metabolites and waste products which would kill the cells otherwise. Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, USA) is a widely used cell culture media. It contains around 15 important amino acids, 7 vitamins and 7 inorganic salts. In addition, it contains D- glucose and phenol red which gives it the red appearance. Along with this media we use 10% FBS (GIBCO, USA) as supplement. Cells were cultured in a T-25 flask and maintained for 24-36 hr before fixing. The protocol followed is

1. Glass bottom dishes immersed in alcohol are taken out and air dried. Then they are kept in UV chamber for 20 minutes.
2. Micropatterned coverslips are attached to the bottom of dishes using silica gel. It is observed with extreme care that right amount of silica is used evenly at the bottom so that nothing extra is poured into the center.
3. The flask with cells is taken out from the incubator to the culture hood. Every other required element like pipets, discarding tubes, empty falcon tubes, cell culture media is kept ready for use.
4. The current media is discarded from the flask and cells are washed with PBS. Then 1ml of 0.25% trypsin-EDTA solution is added to the flask and kept undisturbed in the incubator for 10 minutes.
5. After 10 mins the flask is gently tapped in order to make sure all cells are detached from both surface and other cells. Then solution is taken in 15 ml falcon tubes along with 2 ml of media and centrifuged at 3000 rpm for 4 minutes.
6. After the supernatant is discarded, new media is added to the pallet got from centrifugation. Then cells are added to the glass bottom dishes prepared earlier.
7. The dishes are supplemented with extra media and stored in the incubator for around 24hr - 36 hr.

Then separated dishes are selected for different conditions like ezrin inhibition, ezrin transfection, ezrin siRNA transfection and a control in each case.

For ezrin inhibition 40 μM working concentration of ezrin inhibitor is prepared from a stock of 40 mM. Then inhibitor is added to the media and incubated for 1 hr at 37°C. At the end of 1hr cells were fixed.

For ezrin transfection m-Emerald-ezrin-N-14 (plasmid #54090) is used and for ezrin siRNA transfection Anti-ezrin siRNA is used. Transfection was done at least after 12 hrs of

cell seeding and 0.5 µg of plasmid DNA used for transfection by lipofection method using Lipofectamine 3000 reagent (Life Technologies) in all cases. Then dishes were fixed using 4% PFA and stained using either Alexa Fluor 488 phalloidin or Alexa Fluor 568 phalloidin. Phalloidin is a toxin derived from *Amanita phalloides*. Phalloidin binds to the groove present between two actin monomers of F-actin, which helps demarcate F-actin. The protocol we followed is

1. PFA powder is weighed with 4% m/v concentration and mixed in elix water.
2. The solution is put in hot air to dissolve better.
3. Solution is filtered and stored at 4°C.
4. Cell culturing media is discarded, and cells are washed with PBS twice.
5. Then PFA is added to dishes, and they are incubated for 15-20 mins at room temperature.
6. Then extra PFA is discarded, and cells are washed with PBS twice.
7. Phalloidin is added to PBS at a ratio of 1:200 dilutions.
8. The working solution of phalloidin is then added to the cells at the center of glass bottom dishes and incubated at room temperature for 45 min.
9. Then again, the cells are washed 3 times with PBS and finally extra PBS is added to the dishes and used for imaging.

3.5 Image Analysis

Fiji is an open-source image processing software, which is an extension of ImageJ written in Java. It has a lot of editing and analyzing tools which are very helpful in processing, detecting objects of interest in your image and quantifying properties of those ROIs. It also allows to record the steps one uses manually and save it as a java script. That script can then automate the manual processes and increases the speed exponentially.

Stress fibers and Non stress fiber regions or cortical regions are regions of Interest in our case. Fiji is used to select those regions properly in TIRFM images and measure mean intensity and area in each case. Since stress fiber and Cortex actin organize in different manner and vary in actin filament length as well as concentration, they look different in structure and. Therefore, they need different algorithms to analyze each one.

Non stress fiber/Cortical actin filaments are shorter in size and length and form cross linking as well, resulting in a mesh. So cortical region actin looks like concentrated points

called punctas in TIRFM images. To randomly select these regions, 20 pixels × 20 pixels ROI is drawn manually using rectangle tool. Since images we use contain more than 1000 pixels × 1000 pixels, we zoom in to individual pixels using the zoom in feature. Fiji supports zooming up to 32:1 and down to 1:32. Since our images are of 16- bit depth we use 16 times zoom to see individual pixels. After sufficient zoom, a 20×20 rectangle is drawn. After selection of multiple ROIs, they are added to ROI manager of Fiji. ROI manager is a tool to manage ROIs and perform various tasks using them (Figure 6.). One obvious task is to measure different region properties for which there is a measure option in the manager. Other tasks can be add, delete, select, deselect ROIs. One can Flatten those ROIs as overlay on original Image use for representation purpose. Using all these features data is generated and saved for further analysis. The general flowchart is given in the figure 5.

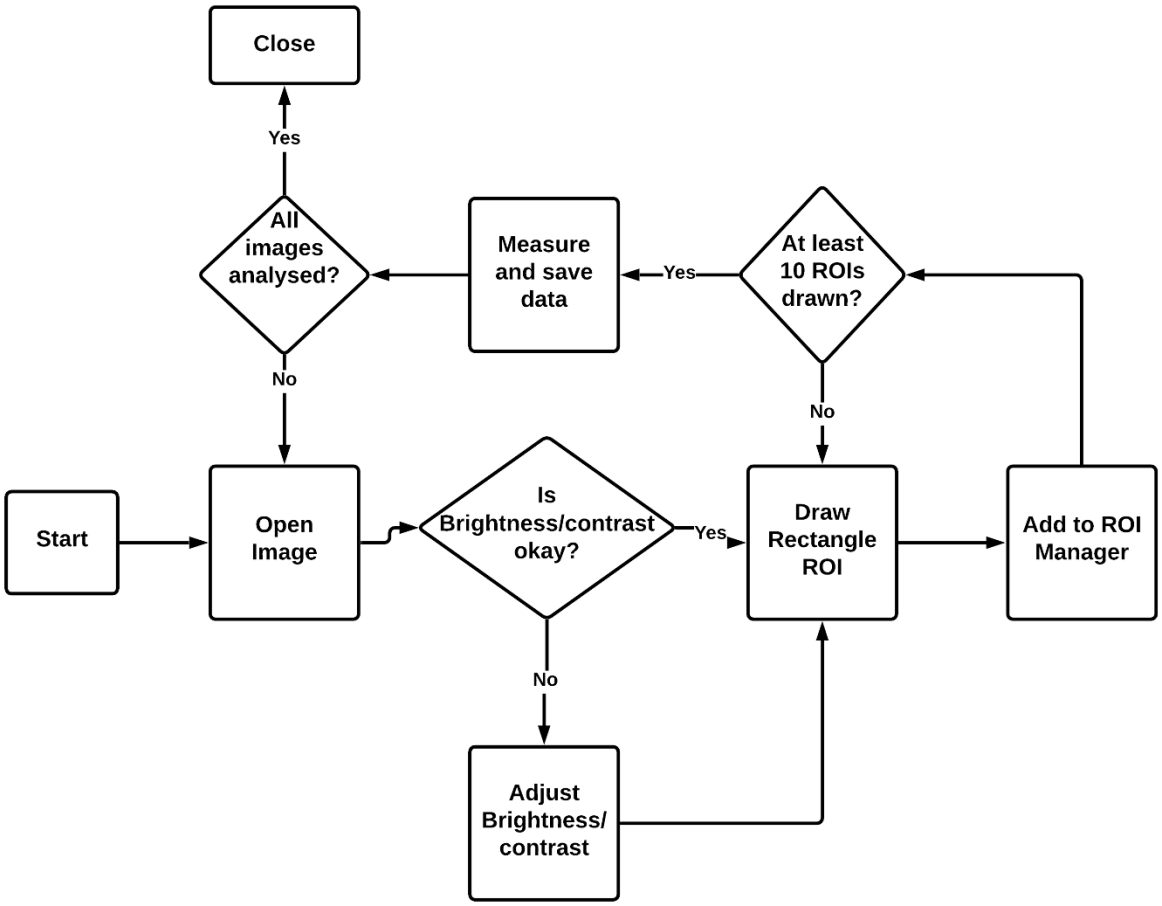

Figure 5 Flowchart describing steps to measure Non stress fiber/Cortical region.

This flowchart is then converted to a macro and used to generate data automatically. But Fiji can't differentiate and select ROIs spontaneously. Therefore, an interactive user interface is generated using specific code (i.e. WaitForUser) to draw ROIs. Then rest of the process is automated. Execution of the code minimises the tedious manual work and helps saving time.

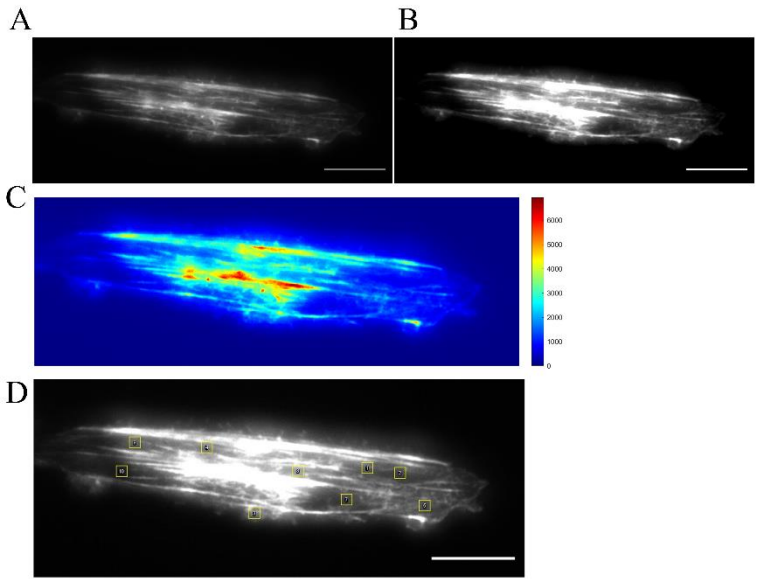

Figure 6 A.TIRF image of well spread CHO cell and patterned to fixed width of 10 µm. B. Shown here is the image with enhanced brightness for better visualization C. Color coded representation. Apparently stress fiber regions show high intensity values. D. Yellow boxes show ROIs selected on cortex regions. Scale bar represents 10 µm.

Stress fibers are formed by long actin filaments bundling together using actin cross linking proteins. Therefore, unlike cortex they look like long linear structures with definite shapes. To detect those objects, Fiji offers a novel method using pixel value or intensity distribution as a defining feature.

Once an image is opened in Fiji, it has values of all the pixels of image as a matrix array. Our images can have value ranging from 0 to 65535. So, it saves a data equivalent to matrix of size of the image with values in the range 0 to 65535. Then it gives the option to select only those pixels, which lie in a particular value range. This method is called thresholding. For example, if we select the threshold to be 50-100 (Figure 7.C), then only pixels with intensity values in that range would be selected.

Thereafter another tool called analyze particles is very crucial in detection of objects. As any physical object would have a definite shape with a defined closed boundary, an image

could also contain two dimensional objects with closed boundary. So, an object is nothing but collection of pixels. Similarly, the stress fibers are collection of pixels in a linear thread like structure. If with proper thresholding we can select out only the pixels in stress fiber region then with analyze particles tool we would be able to find out area, mean grey value and other properties.

Another modification that might help in increasing the contrast of stress fibers is to reduce noise using a tool that does background subtraction (figure 7.B). With proper choice of radius, which would be used to do the subtraction, image contrast can be enhanced greatly. After doing so object detection becomes easier and hence stress fibers are measured properly.

In addition, analyze particle tool gives an option to select objects in desired area range, and with desired circularity (Figure 7.D). Stress fibers are long filaments; therefore, our size range would be medium to relatively infinity. That would help us leave out any noise pixels as they would be smaller in size. Also, we can set our circularity to relatively low as linear structure would have less circularity.

Most important modification required before all these steps is to crop out single cells. After understanding how thresholding works, it would be easy to realize that presence of two cells in a single frame would lead to thresholding of stress fibers in both the cells and that would lead to ambiguity of identity. Therefore, cells are cropped out using rectangular ROI

Using all these features stress fibers are detected. Depending on signal strength, as it varies from image to image, number of stress fiber filaments detected also vary. To statistically verify the distribution properties, more than 50 cells are imaged and processed. The flowchart is described in the figure 8.

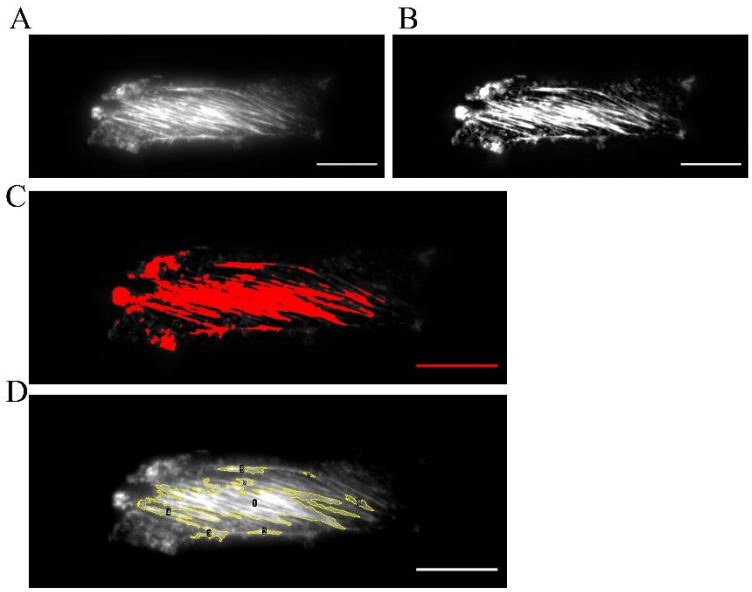

Figure 7 A. TIRF image of well spread CHO cell with fixed width of 10 µm. B. Same image with enhanced contrast by subtraction of background. C. Thresholded image to detect stress fiber regions. D. ROIs detected on stress fiber regions using object detection method. Scale bar represents 10 µm.

Automating stress fiber detection requires user input to threshold image. This is done in a way similar to non stress fiber user interface code (Figure 8). Result files are saved and further analyzed using MATLAB.

MATLAB stands for matrix laboratory. Since any data set can be represented as matrices, MATLAB comes as a great tool to analyze and plot data sets easily. The result files generated using Fiji macros are imported into MATLAB editing space and then they are analyzed to find out statistical values like mean, median, standard deviation, variance etc.

Every data file when imported to MATLAB space is saved as an matrix array by default. There are different data types in MATLAB: numeric types, strings and character, date and time, categorical arrays, tables, timetables, structures, cell arrays, handles etc. Each one has unique usage. The data file we import into MATLAB space is saved as a table.

Table data type has many useful properties. Important ones are : Description contains description of data table saved in a string, User data contains user data saved as an array, DimensionNames saved in a cell as ‘rows’ for row and ‘variables’ for column, VariableNames contains column headings in a cell, VariableDescriptions contains description of variables and is saved as string in cell array, VariableUnits contain units of variable saved in a cell as string array etc.

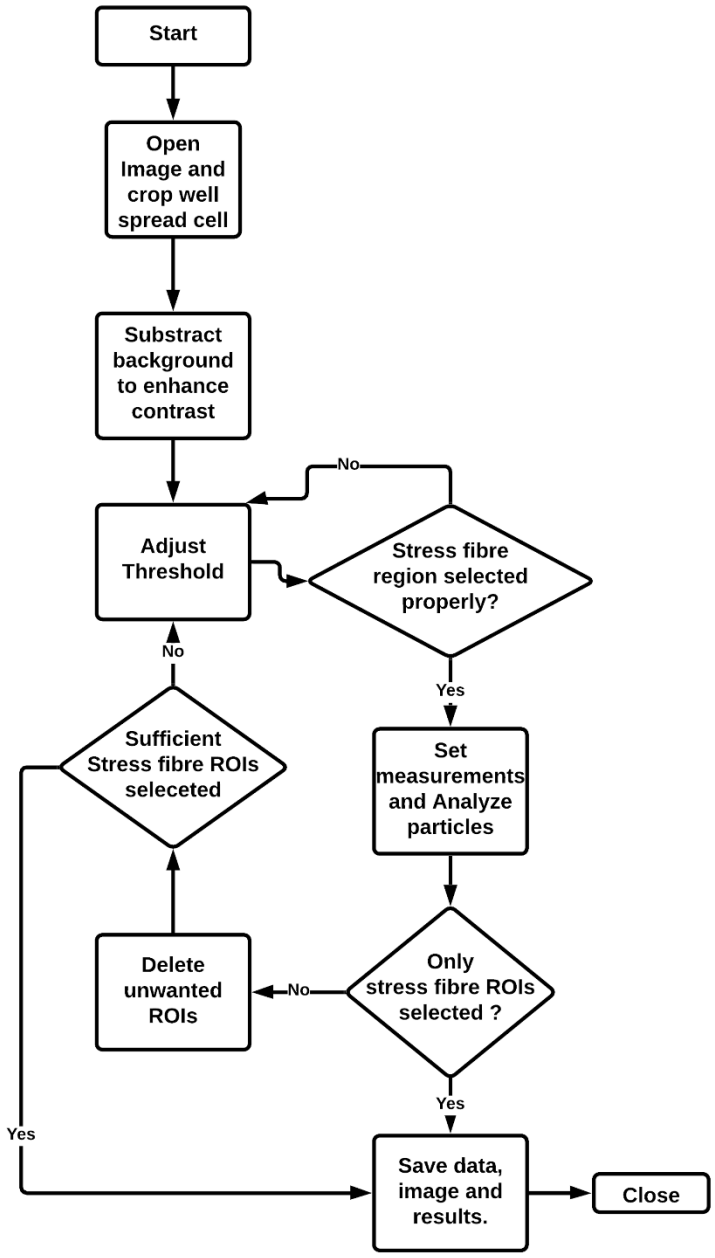

Figure 8 Flowchart description of stress fiber detection and measurement.

The data file imported from results of Fiji processing contains a table with 3 columns. Number of rows varies as it depends on the number of ROIs selected. The columns represent serial number, area and mean gray value with headings as '{}', 'Area', 'Mean' respectively. Serial number heading goes empty as Fiji doesn't provide a heading name. The 'Area'

represents number of pixels present inside each ROI which maps to size of structures of our interest. As described earlier each pixel has a value which is also called the gray value for monochrome images. This value actually represents intensity map of images captured through microscopy. The map can be derived as follows. Each fluorophore is tagged to individual F-actin grooves. Number of fluorophores tagged would be proportional to the amount of F-actin. If we assume each fluorophore emits one unit of intensity, then amount of intensity would be proportional to amount of F-actin. Accordingly, gray values represent the amount of F-actin present. So, mean gray value represents, mean of intensities of pixels present in ROI structure. That maps to mean amount of F-actin present in the ROI.

Each cell would have many ROIs for both stress fiber and cortical region. To find out mean amount of F-actin in a single cell we need to calculate total number of pixels inside the structures of our choice, also total intensity values of those pixels and divide the latter with the former. Therefore, we need to multiply the individual means to corresponding area and sum them up. MATLAB table property comes handy here. We can directly select the area column of the table by '.' extension, for example data.Area gives us the column array of area. Similarly, after selecting mean value, we can perform element wise multiplication by the command 'data.Area.*data.Mean'. The '.' tagged at the end of 'data.Area' is necessary to perform element wise multiplication. We also sum up the area column to find out total area. Then divide the two to find mean intensity of F-actin in a cell. This algorithm is run in loop to find mean F-actin intensity in each cell. A new data set with number of rows same as the number of cells and one column with mean intensity values is saved. The flowchart executed is the one described in the figure 9.

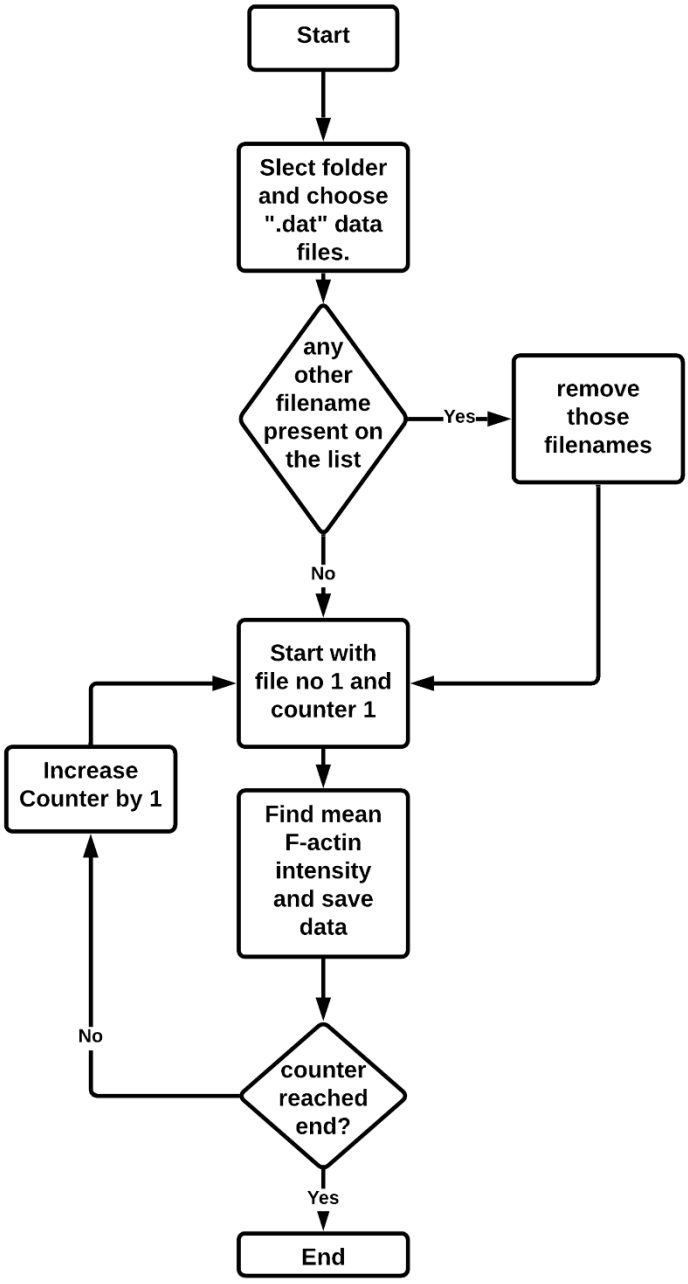

Figure 9 MATLAB algorithm for processing data files in batch

4 Results

This chapter discusses the key results of this study. Some background results that were confirmed in the lab (Thesis (Rinku Kumar, BS Lab, IISER Kolkata)) are:

1) Ezrin inhibitor reduces phospho-Ezrin in cells (Fluorescence-Activated Cell Sorting (FACS))
2) Ezrin activation by EGF increases phospho-Ezrin in cells (FACS)
3) Ezrin inhibition enhances traction forces in cells (Traction Force Microscopy)
4) EGF reduces traction stress (Traction Force Microscopy)
5) Ezrin transfection creates a mesh-like structure (TIRF)

4.1 Demarcating stress fibers and cortical actin

TIRF images clearly show high signal to noise ratio and analyzing signal distribution by plotting histogram shows presence of two peaks implying cortical and stress fiber actin abundance.

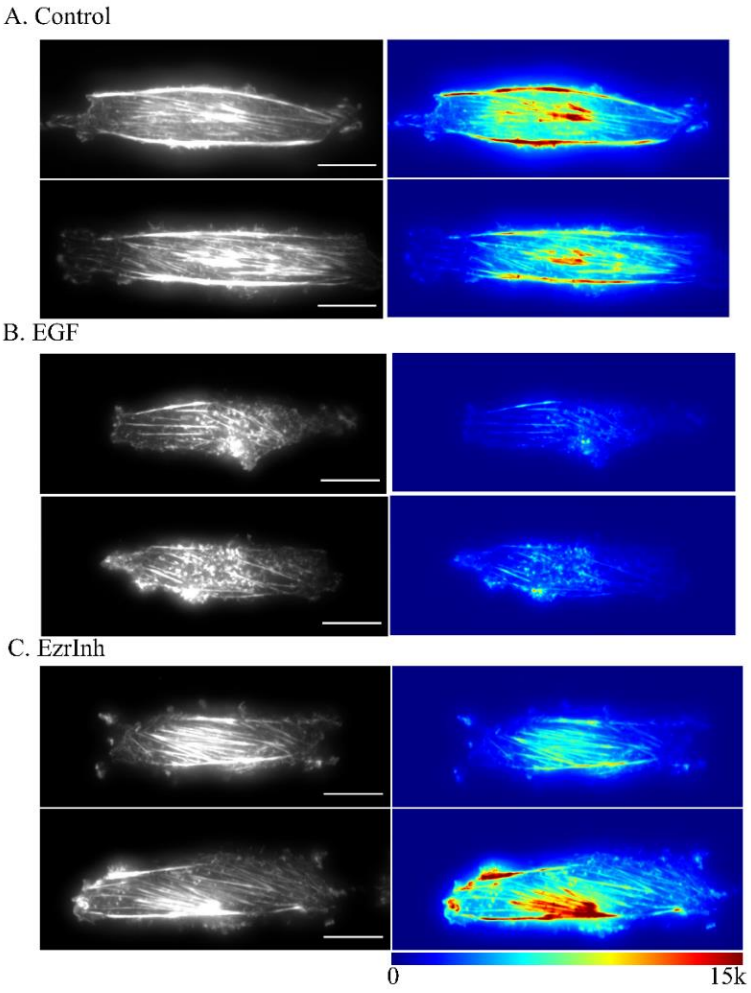

Figure 10 A. CHO control cells with corresponding color (Jet Map) representation. B. EGF treated CHO cells. C. Ezrin inhibited CHO cells and corresponding color representation. The scale bars represent 10 µm.

4.2 Total basal f- actin concentration show changes in ezrin inhibited cells

Ezrin, as it forms linkage between the membrane and cortex, affects both of them if altered. And studies have shown that it regulates membrane tension and cortical tension. So, in ezrin inhibition, ezrin would be inactivated and hence can't form the linkage. So, we expect there to be a change in actin concentration because both membrane tension and cortex tension regulates actin distribution.

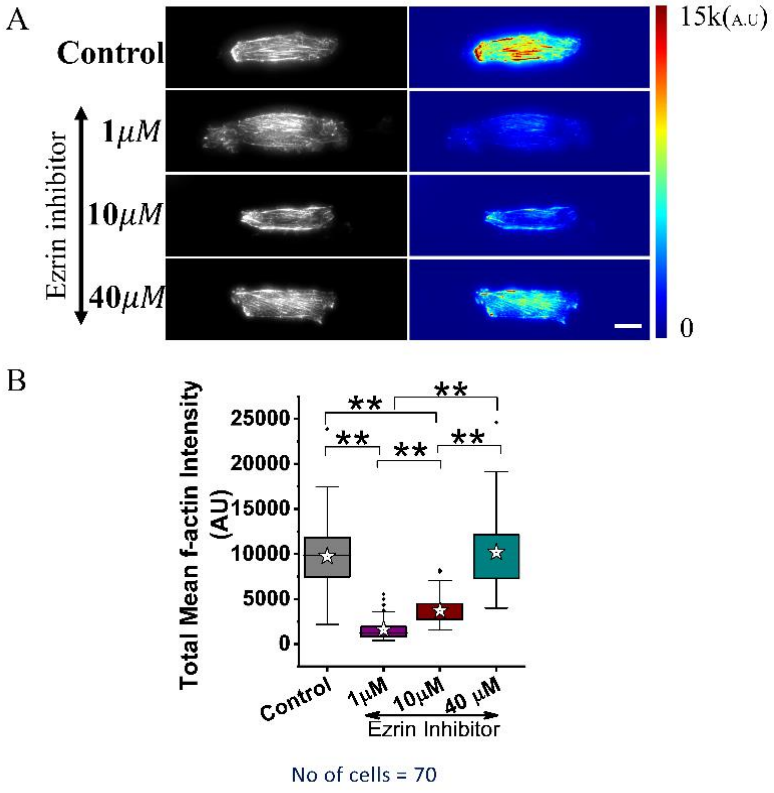

Figure 11 A. Color representation shows change in f-actin distribution in ezrin inhibited cells. B. Boxplot of mean f-actin intensity. Scale bar represents 10 µm.

Result shows change in f-actin intensity is different for different doses of concentration. we further investigate how exactly it affects f-actin distribution with keeping ezrin inhibitor concentration constant at 40 µM which.

4.3 EGF reduces basal mean f-actin concentration.

EGF, as observed from another study in lab, increases phospho-ezrin (pEzrin) level. And pEzrin as is unmasked and activated, forms linkage between the actomyosin cortex and membrane as well as the actin filaments present in stress fibers and membrane. Myosin II with actin filaments generates contractile forces and pulls actin inward. Generally, that leads to decrease in membrane tension and since number of linkages increased due to increase in pEzrin level the membrane tension would tend to decrease more. And keeping in mind that the time duration is very short cell must respond fast to maintain the membrane homeostasis. Result shows basal mean f-actin concentration decreases

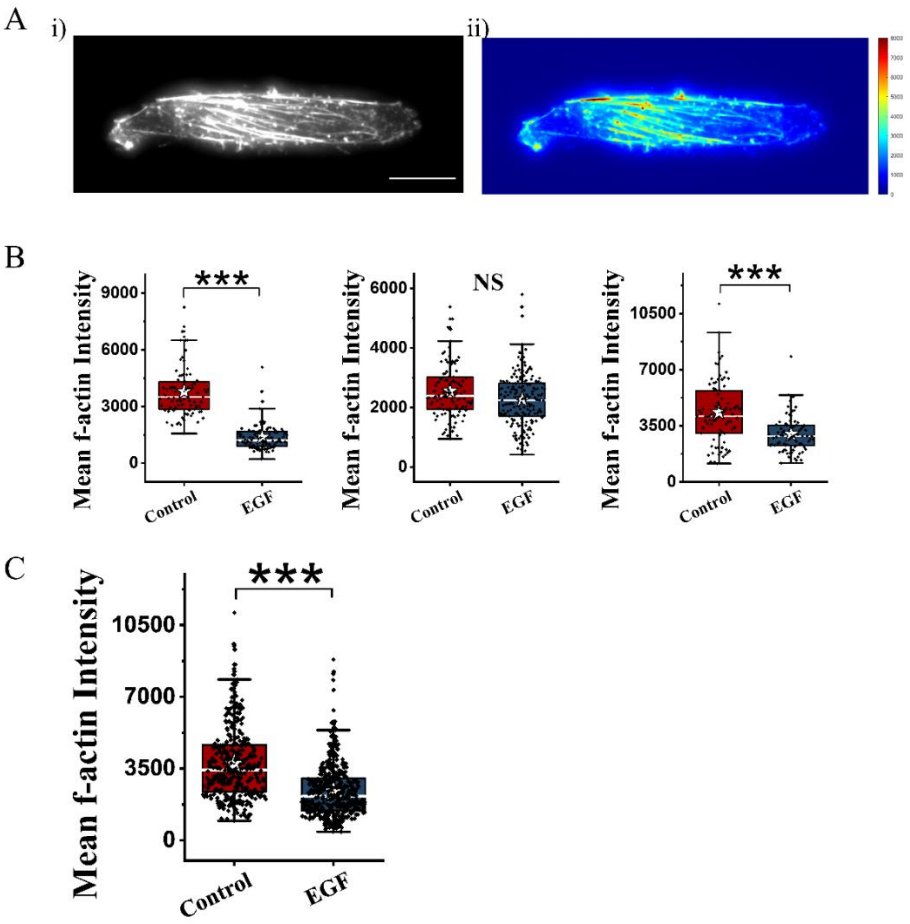

*Figure 12 A. i) TIRF image of micropatterned EGF treated CHO cell ii) color (Jet Map) representation of same cell. B. Boxplot of mean f-actin intensity of control and EGF treated cells for 3 repeats with $n_{control}$=80 cells and n_{EGF} =105 cells, $n_{control}$=110 cells and n_{EGF}=171 cells, $n_{control}$=82 cells and n_{EGF}=78 cells from left to right. F-actin intensity is decreasing in the 2nd repeat though not significant. ***p<0.005, Wilcoxon signed rank test C. Collated data boxplot for $n_{control}$=273 cells and n_{EGF}=354 cells. Central line in boxplots represents median and star represents mean. The scale bar represents 10 µm.*

Since reduction of actin concentration in stress fiber would lead to decrease in contractile force, that would decrease the traction force, which is also confirmed in another study in our lab.

4.4 Ezrin inhibition results in increase of basal stress fiber and stress free f-actin intensity.

Similarly, ezrin inhibitor would inactivate ezrin and concentration of pEzrin would decrease.

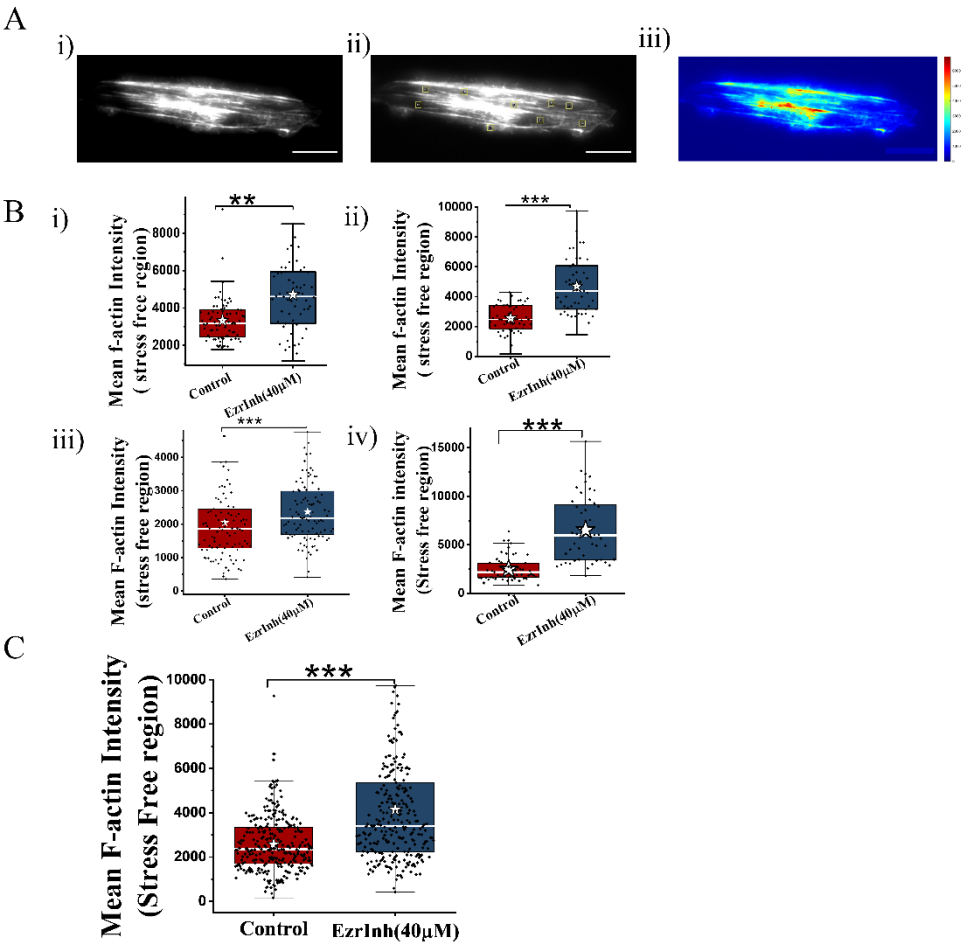

Figure 13 A. i) TIRF image of micropatterned CHO cell. ii) ROIs selection on cortical region. iii) Color (jet colormap) representational image of CHO cell. B. Boxplot of mean f-actin intensity of 4 repeats with i) $n_{control}$ = n_{ezrinh} = 78 cells. ii) $n_{control}$ = n_{ezrinh} = 51 cells. iii) $n_{control}$ = 98 cells, n_{ezrinh} = 100 cells. iv) $n_{control}$ = 53 cells, n_{ezrinh} = 48 cells. C. Collated data boxplot with $n_{control}$ = 277 cells, n_{ezrinh} = 280 cells. The scale bar represents 10 μm.

That would result in lower linkages in MCA and hence the membrane tension would increase. Here also the time duration is less, so to come back to homeostasis, membrane has

to decrease its tension. In this scenario, result shows significant increase in cortical actin region, The statistical values for non-stress regions shows that in ezrin inhibited cells mean f-actin increases around **1.45 fold**, as shown in figure 13 and table 1.

Table 1 Statistical values for control and ezrin inhibition.

Sl no	Control median	Ezrinh median	p value
1	3229	4607	0.0002
2	3405	6069	10^{-8}
3	1586	2188	0.007
4	2314	5977	$3*10^{-9}$
5	2351	3401	10^{-20}

Similarly in stress fiber regions also, result shows a significant increase in actin concentration. The statistical values for stress regions show that in ezrin inhibited cells mean f-actin increases around **1.66-fold**, as shown in figure 14 and table 2. So, result shows that stress fiber region is more strengthened than cortex in ezrin inhibited cells. Further analyzing total stress fiber intensity and ratio of stress fiber to cortical actin (Figure 15), Small but significant increase in stress fiber to cortex ratio proves stress fiber strengthening is more. That leads to another question that how actin distribution increases in both areas but more in stress fiber. To answer this, we look if the cortex/stress fiber is affected by other factors. One study in our lab states that traction force and spread area regulates cortex thickness. Here we already know that traction force increases in ezrin inhibited condition. Along with that we also used ezrin siRNA transfection to further understand what the effect of total ezrin knockdown is as inactive ezrin is also involved in some regulation as described earlier.

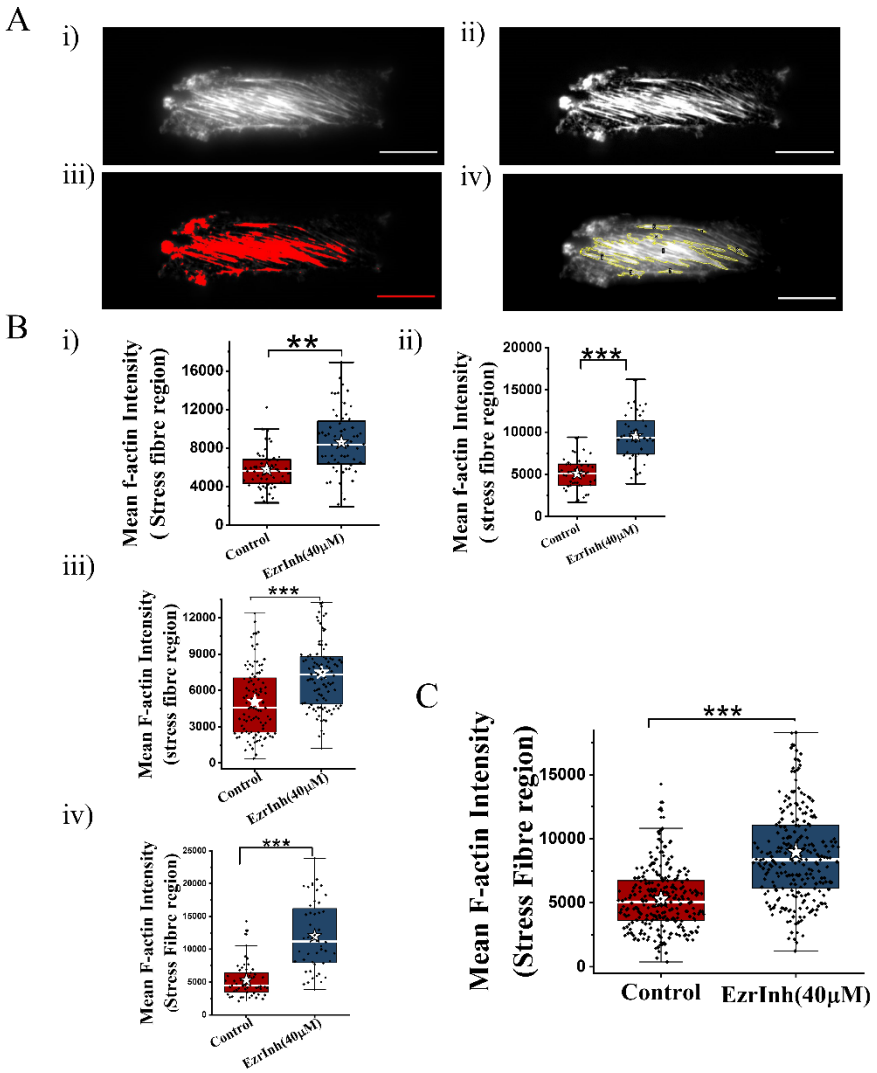

Figure 14 A. i) TIRF image of micropatterned CHO cell. ii) ROIs selections on stress fiber region. iii) Color image of CHO cell. iv) Stress fiber detection with ROIs overlayed on those fibers. B. Boxplot of mean f-actin intensity of 4 repeats with i) $n_{control}$ = n_{ezrinh} = 60 cells. ii) $n_{control}$ = 47 cells, n_{ezrinh} = 50 cells. iii) $n_{control}$ = 98 cells, n_{ezrinh} = 100 cells. iv) $n_{control}$ = 53 cells, n_{ezrinh} = 48 cells. C. Collated data boxplot for $n_{control}$ = 258 cells, n_{ezrinh} = 241 cells. The scale bar represents 10 μm.

Table 2 Statistical values for stress fiber f-actin intensity of control and ezrin inhibition

Sl no	Control median	Ezrinh median	P value
1	5591	8377	0.000013
2	5127	9306	10^{-7}
3	4566	7330	10^{-6}

4	4472	11277	$4*10^{-9}$
5	5084	8390	10^{-25}

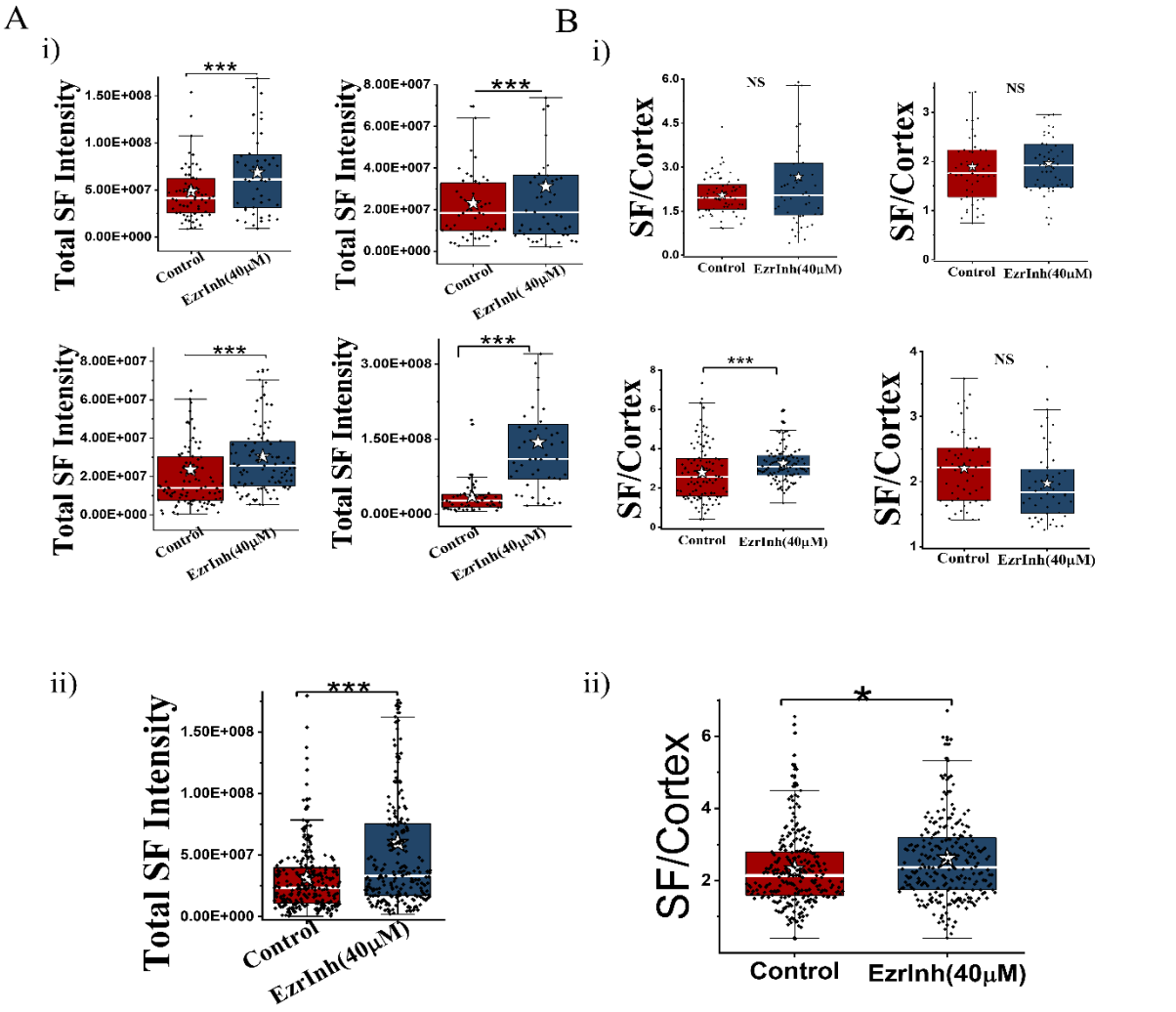

Figure 15 A). i) Boxplot of Total stress fiber intensity for 4 repeats. (upper) $n_{control}$=62 cells and n_{ezrinh} =46 cells, $n_{control}$=49 cells and n_{ezrinh}=51 cells, (lower) $n_{control}$=102 cells and n_{ezrinh}=96, , $n_{control}$=53 cells and n_{ezrinh}=48 cells from left to right in each row. ii) Boxplot for total stress fiber intensity with all data collated. B). i) Boxplot of stress fiber (SF) to cortex ratio for 4 repeats. (upper) $n_{control}$=62 cells and n_{ezrinh} =48 cells, $n_{control}$=50 cells and n_{ezrinh}=51 cells, (lower) $n_{control}$=98 cells and n_{ezrinh}=100, $n_{control}$=53 cells and n_{ezrinh}=48 cells from left to right in each row. Ratio is increasing in repeat 1 and 2 although not significant. ii) Boxplot for stress fiber (SF) to cortex ratio with $n_{control}$=263 cells and n_{ezrinh}=297 (all data collated).

4.5 Ezrin siRNA transfection shows different effect than ezrin inhibition.

We observed that in siRNA treated mean f-actin intensity doesn't increase.

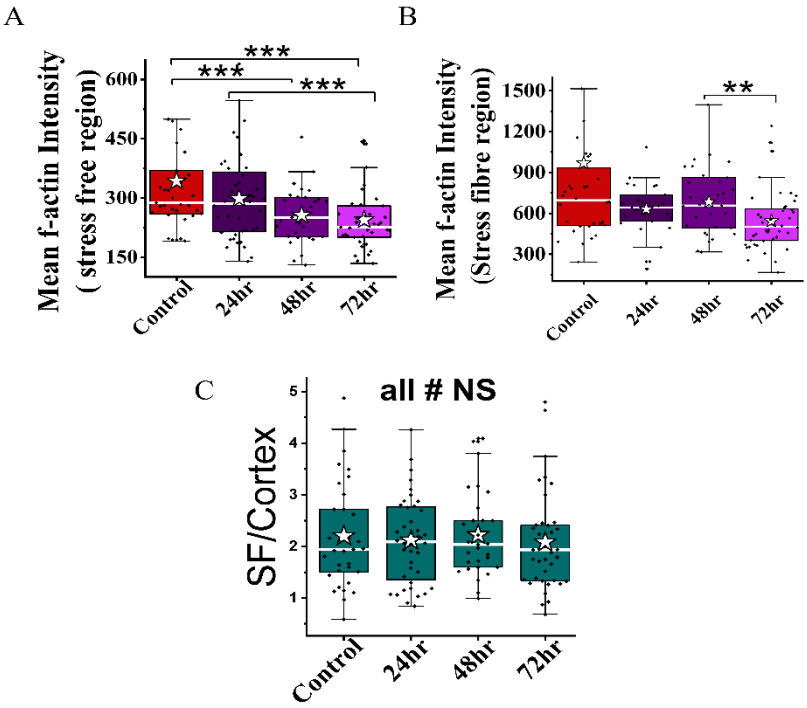

Figure 16 A. Boxplots for stress free regions with $n_{control}$=35 cells, n_{24hr}=40 cells, n_{48hr}=29 cells and n_{72hr} = 39 cells. B. Boxplots for stress fiber regions with $n_{control}$=32 cells, n_{24hr}=35 cells, n_{48hr}=31 cells and n_{72hr} = 44 cells. C. Boxplots for stress fiber and stress free ratio with $n_{control}$=35 cells, n_{24hr}=40 cells, n_{48hr}=29 cells and n_{72hr} = 39 cells.

Stress fiber to cortex ratio also shows no significant difference suggesting both observe same change.

4.6 Spread area distribution in ezrin inhibition.

Interestingly, spread area distribution shows small but significant decrease. Since spread area regulates cortical distribution, this shows that ezrin inhibition and cortical distribution is correlated.

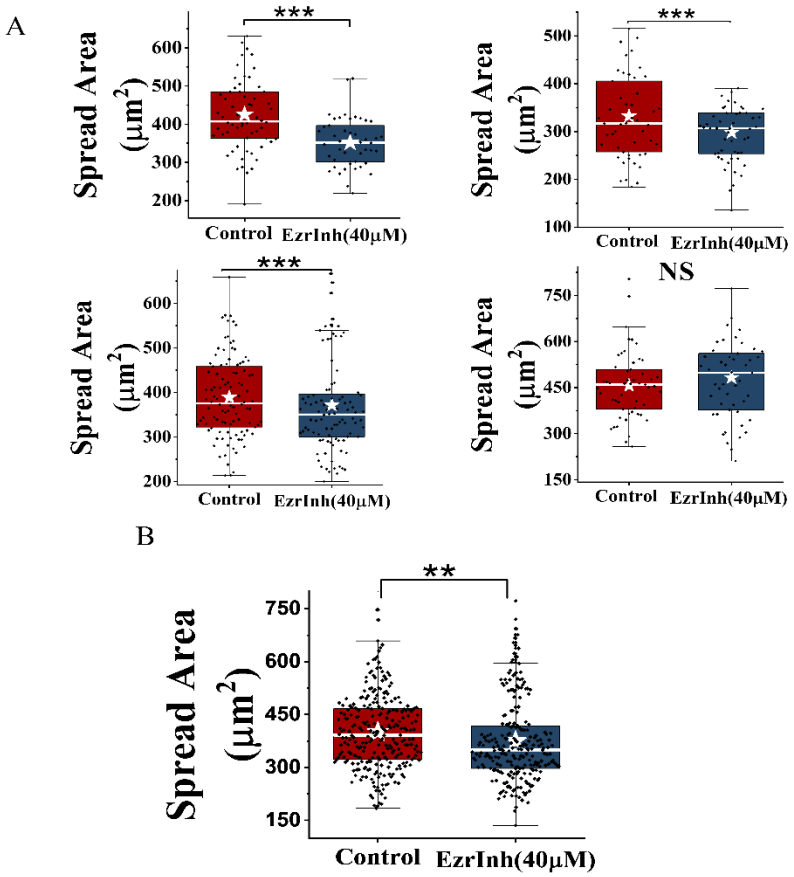

Figure 17 A. Boxplot for spread area of cells in 4 repeats. (upper) $n_{control}$=62 cells and n_{ezrinh} =46 cells, $n_{control}$=49 cells and n_{ezrinh}=51 cells, (lower) $n_{control}$=102 cells and n_{ezrinh}=96, , $n_{control}$=53 cells and n_{ezrinh}=48 cells (from left to right in each row). B. Collated data boxplot of 266 control and 241 ezrin inhibitor cells.

5 Discussion and Conclusion

Ezrin is a multifunctional protein as described earlier. It regulates both membrane and cortical tension. Also, it regulates growth cones, filopodia, etc. by controlling actin polymerization. One of the regulatory pathways in which ezrin is believed to have a role is Rho signaling pathway and people have reported about Rho pathway regulating actin polymerization and FA formation[47,48]. Hence ezrin holds possibility of regulating actin polymerization in more ways. Ezrin inhibition leads to increase in both stress fiber and cortical region. So, to address this question we also take membrane into account as it is an inevitable and important constituent in MCA. Numerous studies have shown, membrane is sensitive to mechanical perturbation and maintains homeostasis to keep cellular shape, integrity and processes intact.[49] it is known that inward pulls by myosin II reduces membrane tension and also myosin II has role in changing hydrostatic pressure which also affects membrane tension. To maintain homeostasis membrane reacts in various ways such as it alters actin polymerization[50,51] which leads to change in membrane tension by formation of various structures like stress fiber, FA_s, cortex acto-myosin mesh etc. or studies also show membrane tension also regulate Rho signaling pathway[52] leading to formation of actin structures or membrane fusion leads to balancing of membrane tension as elastic energy is exchanged during exocytosis or endocytosis.[53]

Ezrin inhibition increases traction force and that is confirmed by increases in stress fiber intensity and also increases membrane tension by decreasing MCA linkages. And keeping in mind that this happens in a time window of 1 hr which is relatively short, membrane would react instantaneously to maintain homeostasis. Here we propose that this abrupt change in membrane tension leads to formation of stress fibers by signaling pathways like Rho or possibly any other pathway (Figure 18). We also speculate that role of ezrin here is not limited to only link cortex and membrane but it might also be involved in activation of Rho pathway which needs further investigations. Similarly, EGF treatment showed that mean basal f-actin intensity decreases as EGF increases phospho-ezrin level (confirmed result in lab) and that leads to decrease in membrane tension as opposite to ezrin inhibition. Decrease in f-actin intensity might be regulated by deactivation of Rho pathway which might again involve ezrin. Therefore, all these clues points finger in the direction of ezrin mediated regulation. Another study from our lab describes that traction force regulates cortical thickness at the periphery of cells other than basal cortex. Increasing traction force decreases cortex thickness. But decrease in spread area increases cortex thickness. Since, here result shows that cortical actin distribution

also increases in the basal plane, further investigation on what happens to total f-actin distribution becomes instrumental for proper explanation. Observing study done in our lab using flow cytometry experiment to observe total f-actin showed that total intensity doesn't change in ezrin inhibited cells with respect to control cells.

Therefore, these investigations clearly show that f-actin is redistributed between internal f-actin poll and the stress fiber and cortex region and that leads to increase in both stress fiber and cortex region. But it is more in stress fiber region.

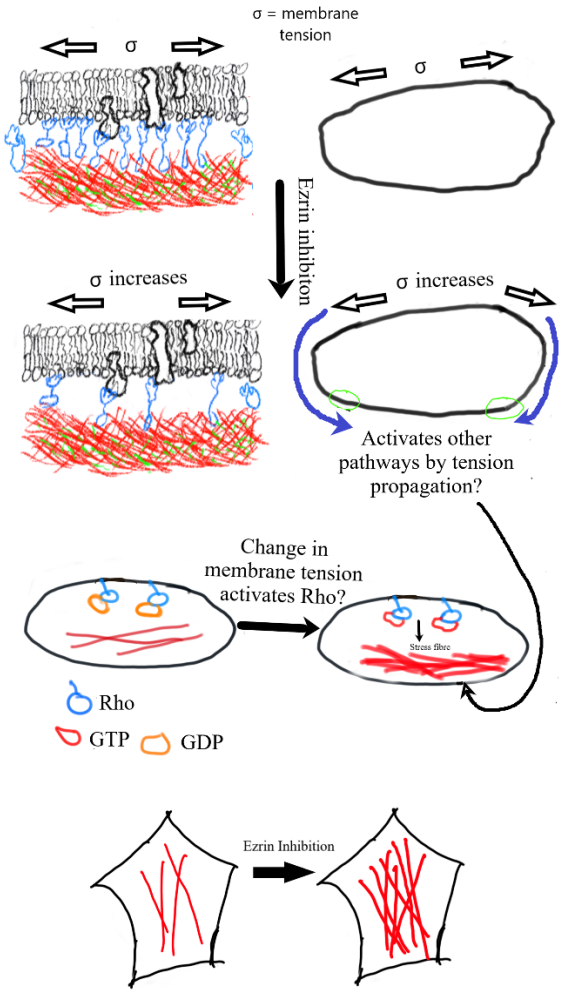

Figure 18. Cartoon Schematics describing possible pathways that lead to stress fiber formation by ezrin inhibition.

Thus, examining further we note that area decreases slightly in ezrin inhibited cells. Since the same study which examined cortical thickness regulation, showed that decrease in area leads to increase in cortical thickness, it is a possibility that traction force and spread area both play a similar role in case of ezrin inhibition. Both balance out the amount of cortical actin

intensity regulated by ezrin and resulted in net increase of cortical actin intensity but less than stress fiber.

To understand further the role of ezrin, when total ezrin concentration is decreased by ezrin siRNA treatment, it showed a different effect than ezrin inhibition raising further questions about how ezrin works. There are many possibilities at this point like cell behaves differently in longer time duration as it gets enough time to for maintaining homeostasis. And also when ezrin has been knocked out totally it has a different effect than when in inactive form. Considering all these, it is clear that ezrin holds a lot of regulatory possibilities implying its importance in MCA.

6 Limitation

Stress fiber detection method using local thresholding based segmentation underestimates the intensity of stress fiber regions as it also contains some cortical regions. Therefore, it limits the estimation of stress fiber measurement. Another developed algorithm holds the possibility of properly identifying stress fiber structures which would lead to better estimation of stress fiber intensity and thus would increases the clarity in results. The method used in previous analysis versus the better algorithm is described in the figure 19.

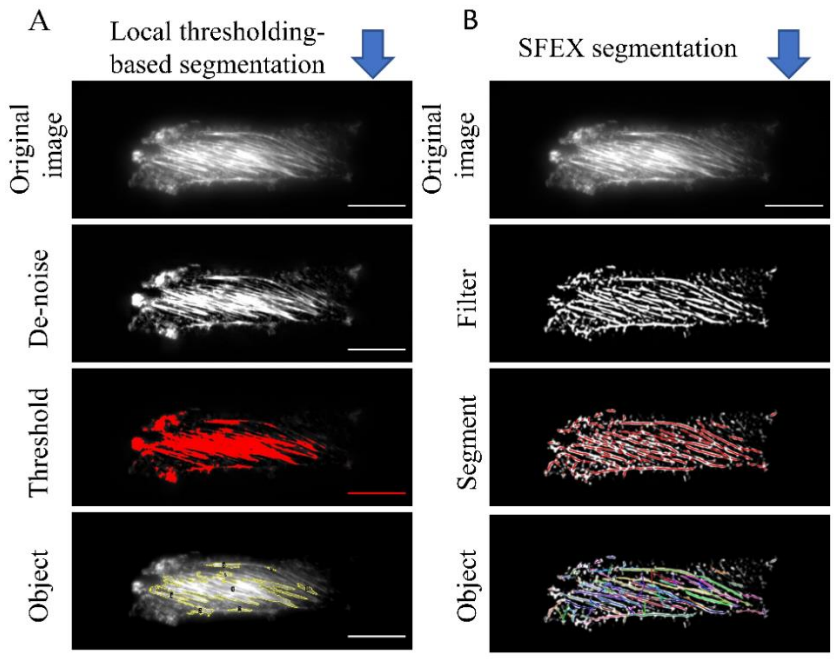

Figure 19 A. Process describing stress fiber detection using manual thresholding B. Process describing stress fiber detection using Stress Fiber Extractor (SFEX)[54] algorithm. Note that method B has higher precision.

7 Future perspectives

Since myosin is key to cortical stiffness and regulates cortical actin distribution it is also an important molecule which is involved in ezrin regulating f-actin distribution in stress fiber and cortical regions. It has been also shown in the cortical thickness regulation study that myosin turnover rate change leads to cortex thickness regulation.[44] Therefore, future investigation on how turnover rate of myosin II changes during ezrin inhibition would help in explaining the exact mechanism of regulation.

Cortical stiffness or cortical tension is also a crucial parameter which is key to cortex regulation. Therefore, study on how cortical stiffness is changing during ezrin inhibition would shed more light on increase in basal cortical f-actin distribution during ezrin inhibition.

Ezrin is involved in other regulatory pathways in both active and inactive form, therefore insight into how these signaling pathways respond to ezrin inhibition would lead to more clarity.

Lastly membrane tension regulation also can initiate many regulatory pathways whose domains are present on membrane and are tension sensitive. Study on those areas would also open up new possibilities and understanding to role of ezrin.

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imref$_1$- https://www.researchgate.net/publication/235132960_On_the_Potential_Use_of_Evolutionary_Algorithms_for_Electro-Optic_System_Design/figures

imref$_2$ - https://micro.magnet.fsu.edu/primer/techniques/fluorescence/tirf/tirfintro.html

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9 Appendix

9.1 Measuring stress fiber intensity in batch (FIJI)

```
directory = getDirectory("choose directory");
print(directory);
filelist = getFileList(directory) ;
```

```
for (i = 0; i < lengthOf(filelist); i++) {
   if (endsWith(filelist[i], ".tif")) {
      open(directory + File.separator + filelist[i]);
      filename = File.getName(directory + File.separator + filelist[i]);

selectWindow(filename);
run("Stack to Images");
waitForUser("select the window");
run("Subtract Background...", "rolling=50");
setAutoThreshold("Default");
\\\\run("Threshold...");
setThreshold(4009, 8634);
selectWindow("Threshold");
title = "Setting the Threshold";message = "set threshold properly";
waitForUser(title, message);
\\\\setTool("rectangle");
run("Analyze Particles...", "size=500-Infinity circularity=0.00-0.55 display clear add");
roiManager("Deselect");
close();
open(directory + File.separator + filelist[i]);
selectWindow(filename);
run("Stack to Images");
waitForUser("selectwindow");

saving_dir = "\\Users\\";
name = "CHO_phalloidin_A488_"+i;

savename = saving_dir+name+"_RoiSet.zip";
roiManager("Save", savename);
run("Clear Results");
roiManager("Show All");
roiManager("Measure");

savename2 = saving_dir+name+"_Results.dat";
saveAs("Results", savename2);
roiManager("Deselect");
roiManager("Delete");
close();
print(i);
}
}
```

9.2 Measuring cortical region in batch. (FIJI)

```
directory = getDir("choose directory");
filelist = getFileList(directory)


for (i = 0; i <= lengthOf(filelist) ; i++) {
   if (endsWith(filelist[i], ".tif")) {
      open(directory + File.separator + filelist[i]);
      filename = File.getName(directory + File.separator + filelist[i]);

selectWindow(filename);

Dialog.create(" Want to use the image");
         Dialog.addCheckbox("No", true);
   Dialog.show();
   if(Dialog.getCheckbox()==true){
         close("*");
         continue
   }
title = "Selecting ROIS";message = "Select ROIS at stress free region";
waitForUser(title, message);

saving_dir = "/Users/amrutamaya/Google Drive/Role of Ezrin in MCA/Analysis/siRNA/Non
stress fiber_Intensity/72hr/";
name = "CHO_phalloidinA488_"+i;

savename = saving_dir+name+"_RoiSet.zip";
roiManager("Deselect");
roiManager("Save", savename);
roiManager("Show All");
roiManager("Measure");

savename2 = saving_dir+name+"_Results.dat";
saveAs("Results", savename2);
roiManager("Deselect");
run("Clear Results");
close();
}
}
```

9.3 Measuring spread area in batch. (FIJI)

```
directory = getDirectory("choose directory");
filelist = getFileList(directory) ;

for (i = 0; i < lengthOf(filelist); i++) {
    if (endsWith(filelist[i], ".tif")) {
        open(directory + File.separator + filelist[i]);
        filename = File.getName(directory + File.separator + filelist[i]);

selectWindow(filename);
setAutoThreshold("Default");
//run("Threshold...");
setThreshold(4009, 8634);
selectWindow("Threshold");
title = "Setting the Threshold";message = "set threshold properly";
waitForUser(title, message);
run("Analyze Particles...", "size=10000-Infinity display clear");

saving_dir = directory+"/spread.Area/";
name = getTitle();

savename2 = saving_dir+name+"_Results.dat";
saveAs("Results", savename2);
run("Clear Results");
close();
    }
}
```

9.4 Data file processing in batch (MATLAB)

```
message = 'choose the folder with data';
uiwait(msgbox(message));
dir_path = uigetdir();
cd(dir_path);
files = dir('*.dat');
cd(root_dir);
filenames = {files.name};
filenames = filenames(~ismember(filenames,{'.','..','.DS_Store','Mean_int_respective.csv'}));
mean_int_data = zeros(length(filenames),1); area_data = zeros(length(filenames),1);
total_data = zeros(length(filenames),1);
Area_all = []; Mean_all=[]; intensity=[];
```

```
for i = 1:length(filenames)
    current_file = fullfile(dir_path,filenames{i});
    data = readtable(current_file,"PreserveVariableNames",true);
    intensity = data.Mean;
    area = data.Area; Area_all = [Area_all;area];  %#ok<AGROW>
    mean = data.Mean; Mean_all = [Mean_all;mean]; %#ok<AGROW>
    totalint = data.IntDen; intensity = [intensity;totalint]; %#ok<*AGROW>
    total = sum(area.*intensity);total_data(i) = total;
    mean_int = total/sum(area);
    mean_int_data(i) = mean_int;
end
DataName= strcat('Total_respective.csv');
DataFolder=fullfile(dir_path,DataName);
writematrix(mean_int_data,DataFolder);
```

9.5 Mean Basal Intensity (MATLAB)

```
Dir= 'D:\Paper2\Day1_2_Control_EGF_EzrInh\Oly100X_201122_CHO_EGF_EzrInh_f-
actinTIRF_\D2_Control1_';
RootDir=pwd;cd(Dir);
DirFiles=dir('*tif');
DirFile={DirFiles.name};
Filename=DirFile(~ismember(DirFile,{'.','..'}));
for i=1:length(Filename)
    Value=1;j=0;
    while Value==1
        j=j+1;
DataName= fullfile(Dir,Filename{i});
Im= imread(DataName);imshow(Im,[]);
h=drawpolygon();
BWlogic=createMask(h);
close;
I = mat2gray(Im);
Imask=I.*BWlogic;
t=graythresh(Imask);
BW = im2bw(Imask,t);
SegImg=uint16(BW).*Im;
SegImgArray=SegImg(:);SegImgArray(SegImgArray==0)=[];
Intensity=[max(SegImgArray) median(SegImgArray) mean(SegImgArray)];
% subplot(2,1,1);imshow(Im,[]);
% subplot(2,1,2);imshow(SegImg);
[Count,Bin]=imhist(SegImg);
Count1=Count(2:end);Bin1=Bin(2:end);
% histfit(Count1,255,'normal');
```

```
cd(RootDir);
[fitresult,Gausmean, gof] = createFit(Bin1, Count1);
Finalresult=[Gausmean double(Intensity)];
DataName= strcat('IntensityFittedRaw',num2str(i),'_',num2str(j),'.dat');
DataNamej= strcat('IntensityFittedRaw',num2str(i),'_',num2str(j),'.jpg');
DataFolder=fullfile(Dir,DataName);
DataFolderjpg=fullfile(Dir,DataNamej);
writematrix(Finalresult,DataFolder);
saveas(gcf,DataNamej,'jpg')
Value=input('Enter zero or one ');
   end
end
```

9.6 Gaussian Fitting (MATLAB)

```
function [fitresult,Result, gof] = createFit(Bin1, Count1)
%CREATEFIT(BIN1,COUNT1)
%  Create a fit.
%
%  Data for 'untitled fit 1' fit:
%      X Input : Bin1
%      Y Output: Count1
%  Output:
%      fitresult : a fit object representing the fit.
%      gof : structure with goodness-of fit info.
%
%  See also FIT, CFIT, SFIT.

%  Auto-generated by MATLAB on 19-Nov-2020 17:30:09


%% Fit: 'untitled fit 1'.
[xData, yData] = prepareCurveData( Bin1, Count1 );

% Set up fittype and options.
ft = fittype( 'gauss1' );
opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
opts.Display = 'Off';
opts.Lower = [-Inf -Inf 0];
opts.StartPoint = [7263 2313 1305.83689238107];

% Fit model to data.
[fitresult, gof] = fit( xData, yData, ft, opts );
A1=fitresult.a1;B1=fitresult.b1;C1=fitresult.c1;
```

```
Result=[A1 B1 C1];

% Plot fit with data.
figure( 'Name', 'Guass fit' );
h = plot( fitresult, xData, yData);
legend( h, 'Intensity', 'Guass fit', 'Location', 'NorthEast', 'Interpreter', 'none' );
xlabel( 'Intensity (a.u)', 'Interpreter', 'none' );
ylabel( 'Count', 'Interpreter', 'none' );
grid on
```